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BACTERIAL METABOLISM OF CRUDE PETROLEUM

by



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B.Sc.

A THESIS

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The undersigned certify that they have read, and
recommend to the Faculty of Graduate Studies and Research
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BACTERIAL METABOLISM OF CRUDE PETROLEUM

submitted by Allen M. Jobson, B.Sc., in partial fulfilment
of the requirements for the degree of Master of Science.

DateOctober 12, 1971.....

ABSTRACT

Several mesophilic isolates and mixtures have been enriched and cultured, which show assimilative capabilities when growing on inorganic salts medium containing crude petroleum as the sole carbon substrate.

Studies using a Pseudomonas isolate support the conclusion that under anaerobic conditions petroleum hydrocarbons cannot support the growth of such organisms. Under aerobic conditions, however, significant utilization of the hydrocarbons was reflected by an increase in viable counts.

Experiments were undertaken to study the utilization of whole crude petroleum and petroleum components by a mesophilic soil mixture, which had, itself, been enriched in the presence of crude oil. Growth of this mixture on salts medium + 0.1% crude oil, at 30° resulted in a viable count within the range of $1 - 2 \times 10^8$ cells/ml. Analysis of the residual petroleum, extracted from the culture medium with n-pentane was accomplished using silica gel-alumina gel column chromatography as well as gas chromatography. Growth in the presence of whole crude oil resulted in rapid utilization of n-paraffins up to and including nC_{24} . Utilization of the higher n-paraffins required longer periods of incubation. The isoprenoid moieties within the petroleum (pristane and phytane) appeared to be more refractive to utilization, but these too

were metabolized after 21 days incubation. The aromatic fraction, within the crude petroleum, appeared to be the only other fraction subject to significant and detectable amounts of bacterial alteration.

Studies involving the growth of this mesophilic soil mixture on purified hydrocarbon components showed that only the saturate fraction stimulated growth appreciably beyond control levels.

An isolate of Micrococcus sp. was also tested on salts medium and 0.1% crude oil at 30°. Analysis of the residual oil revealed the fact that the isolate could utilize n-paraffins of all chain lengths with equal facility. Viable count exercises showed that a cell concentration of 2×10^7 viable cells/ml was the upper limit of growth under the stated conditions. This Micrococcus strain also possessed the ability to partially utilize components from within the aromatic fraction of the petroleum.

Psychrophilic mixtures were enriched from two soil samples using crude petroleum as growth substrate. Growth trials, at 4°, on salts medium + 0.1% whole oil, indicated that both mixtures were able to completely emulsify the petroleum and utilize significant amounts of the paraffin component within a 21 day incubation period. Paraffins from nC_{31} onward were utilized more slowly than those of smaller chain length. Again, pristane and phytane proved the most refractive to utilization. Theoretical calcula-

tions also indicated that some aromatic utilization was occurring.

The overall action of the mesophilic and psychrophilic bacterial mixtures and isolates, tended to create an emulsified residual petroleum, possessing a specific gravity greater than 1.0 and a higher aromatic (naphthenic) and asphaltenic character. The properties of this residual oil product bear many similarities to equivalent petroleums found at certain locations within natural oil deposits.

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ABBREVIATIONS

ATP	- Adenosine triphosphate
cm	- centimeter
g	- gram
hr	- hour
l	- liter
M	- molar
mg	- milligram
ml	- milliliter
mM	- millimolar
mm	- millimeter
OV-1	- methyl silicone
μ l	- microliter (10^{-6} l)
μ g	- microgram
rpm	- revolutions per minute
SE-30	- silicone gum rubber (stationary phase)
v/v	- volume per volume
w/v	- weight per volume
W98	- methyl vinyl silicone oil (stationary phase)

INTRODUCTION

It is a well established fact that bacteria can cause deterioration of stored petroleum and petroleum products. Evidence is cited in the literature of spoilage of gasoline in storage tanks, and of petroleum products degrading within months of initial extraction. Evidence is also available of sufficient bacterial growth in jet fuels to cause fouling of injection systems and filters in aircraft.

Until recently, it was thought that such bacterial growth was dependent upon a petroleum-water interface. However work by Japanese researchers and more recently by German microbiologists indicates that bacteria can survive and remain viable in the complete absence of water and in an environment of hydrocarbons.

It has also been presumed, until recently, that petroleum deposits are inert bodies of hydrocarbons not subject to compositional changes after genesis is complete. However, geochemists attempting to relate oils genetically have encountered an increasingly evident problem; namely that of crude oils which apparently have lost portions of their paraffin fraction, leaving them with "naphthenic" character. This can cause two oils to vary quite markedly, when in fact one would predict that they should be genetically related. It is known that crude oil may be altered in the reservoir by several natural phenomena. Oils

maturing in a deep reservoir can be changed to such an extent that they no longer resemble any of their genetically related counterparts which have remained at more shallow depths. These types of changes can be explained and present no severe problems to the geochemist. However, anomalous loss of paraffinic character as mentioned above, does present the geochemist with a difficult question. A few workers have proposed that microorganisms are the agents responsible for the paraffin destruction. They base their speculation on the fact that the altered oils are usually found in relatively shallow reservoirs more easily reached by percolating surface waters. However, at the present time few microorganisms have in fact been isolated from such reservoirs, so the proposals are still based on speculation rather than on fact.

Oils suspected of being microbially altered have been found in the Powder River Basin in Montana, in certain small, shallow fields in Japan, from a wild-cat well in North Africa, and from a field in Saskatchewan. The latter deposit holds some interest for Imperial Oil Limited since they hold leases in the area. As a result of numerous tests and measurements, Imperial geochemists are certain that all oils in this particular field should be genetically related, yet great differences are found. In the south-easterly portion of the field, the oils possess superior refining qualities. Such oils have a low weight percentage of

sulfur, a high A.P.I. gravity (low density), and a high saturates content. As well, the groundwater of this area is highly saline indicating little groundwater flow. On the other hand, however, the oils from the more northwesterly portion of the field show degraded, less desirable properties. Sulfur contents of these oils are up to two and one-half times higher, the A.P.I. gravity is lowered (higher density), and the saturates content is reduced. The groundwaters in this region possess very low salinity levels indicating higher amounts of groundwater flow. Again, as in the other cases, only circumstantial evidence can be given to indicate that a bacterial process may be involved in degradative action in the northwesterly portion of the field.

The objectives of this research project were as follows:

1. To convert a southeasterly oil of superior quality, via a microbial process to an oil of degraded properties similar to those found in the oils of the northwesterly portion of the field.
2. To obtain data concerning which petroleum components microflora are capable of utilizing as a sole carbon substrate.
3. To attempt to achieve bacterial growth in petroleum under psychrophilic conditions. This has a direct bearing on decomposition of oil spills in cold climates.

LITERATURE REVIEW

I. General Observations

In reviewing papers, articles, and books concerned with microbiology as applied to the field of petroleum technology, many of the microbiological processes involved have been deduced or assumed by the geochemist. In the past decade, interest has centred around the biological utilization of a great variety of purified petroleum hydrocarbons. The most complete review of this subject up to 1967 and perhaps even up to the present time was presented by A. C. Van Der Linden and G. J. E. Thijsse (1965). The large amount of knowledge gained in this field as evidenced by the length of the review is due in no small part to the increasing availability of pure hydrocarbon samples, the advent of gas chromatography, the mapping of many of the intermediary metabolic pathways, and also the method of Stanier for the sequential induction of these and other metabolic pathways, (1947, 1950). In as much as this review provides useful data for the microbiologist interested in such fields as production of single cell protein, research on the utilization of purified hydrocarbon substrates still does not answer many of the questions concerning the microbiology of the sedimentary petroleum environment. More precisely, as J. B. Davis states in his text, Petroleum Microbiology (1967), "Certainly, one of

the purposes of the discussions to follow is to stimulate interest and effort with regard to microbiological and biochemical investigations related to petroleum geochemistry."

II. Role of Bacteria in Sedimentary Environments

Microbial activities in sediments are known only in rather general terms, and because a major portion of these activities occurs early in the sedimentary environment, there is a tendency to discount their importance in petrogenesis. Kuznetsov's text entitled Introduction to Geological Microbiology (1963), makes an excellent case for the importance of bacteria in petrogenesis as well as other processes of a geological nature.

The synthesis of hydrocarbons is only one role of microbial flora within the sedimentary environment. The fact that bacteria are responsible, at least in part for petrogenesis has been proven circumstantially as well as by the general observation of an ultimate microbial decomposition or modification of most organic matter in nature. Thus it is the organic matter that escapes complete microbial decomposition or mineralization that yields coal and petroleum. At one time, it was the consensus of opinion that once petroleum was formed, anaerobiosis was such that bacterial activities reached a permanently low level. The earliest evidence to refute such a suggestion came when sulfate reducing bacteria were discovered and later when

they were related to petroleum microbiology.

III. Role of Sulfate Reducers

Since the initial paper concerning the reduction of sulfate by bacteria, written by Beijerinck (1895), the sulfate reducers have been implicated in many processes directly or indirectly pertaining to petroleum geochemistry. As a group, they have a major effect on portions of the sedimentary environment. They cause deposits of massive amounts of sulfur, they actively corrode iron, and they become active in anaerobic environments containing organic matter of plant origin.

It is on this subject that a point of contention arises between the microbiologist and the geochemist. In general, the geochemist accepts the hypothesis that apart from the geological processes involved, petroleum genesis is partially dependent upon microbiological activities. Aerobic bacteria initially degrade all or most of the oxygen-containing materials within organic deposits. This ultimately produces reducing conditions and a greatly lowered oxygen tension. This situation allows a population succession to occur, namely the stimulation of an anaerobic population. These anaerobes continue to metabolize and lower the oxygen, nitrogen and sulfur content of organic constituents. Lipid materials such as sterols, fatty acids, and waxes are negatively enriched, ultimately yielding organic materials in

the source sediments which more closely resembles petroleum. This hypothesis was presented by Hammar (1934), and in large part is still accepted as being true. ZoBell (1944), pointed out the changes in elemental analysis during petroleum diagenesis. Carbon levels rose from 49% in marine sapropel to approximately 85% in petroleum. Comparison of the same two extremes showed the oxygen content falling from 40% to less than 1% and the nitrogen content decreasing from 3% to less than 0.5%. The hydrogen content increased from 5% to 12-13%. All these changes can be brought about in vitro using anaerobic Clostridia, Desulfvibrio, etc., thus microbial implication in petroleum genesis is not incompatible with the opinions shared by geochemists.

Opinions are split however on the role or activity of anaerobic organisms within deposits of mature petroleums. By using in vitro conditions of anaerobiosis, various microbiologists have obtained conflicting results as to utilization of n-alkanes as carbon substrates by anaerobic or facultative bacteria. Initial work by various research groups first of all did show that almost without exception sulfate reducers could be isolated from oil field waters in producing areas. Beck (1947), tested the ability of sulfate reducers to grow and generate hydrogen sulfide in a mineral salts medium with crude oil as a sole source of carbon. The systems were heavily inoculated with the cultures, thus small amounts of hydrogen sulfide generated

were discounted as being contaminant carry-over. Controls of cultures inoculated into salts with lactate as carbon source nearly always yielded significantly more hydrogen sulfide. Only three of all cultures tested showed any significant amounts of hydrogen sulfide generated using petroleum as a carbon substrate. Five of the cultures tested showed apparent effects of oil toxicity. In short, Beck concluded that utilization of petroleum was at best very slow among sulfate reducers.

In 1960, Rosenfeld applied for patent rights claiming the detection of sulfate reducing bacteria in subsurface waters as being an index of petroleum. This reasoning was based on an apparent belief of their hydrocarbon-utilizing ability. The method as listed in the patent does not, however, include hydrocarbon oxidation as a criterion but simply claims the detection of such microorganisms grown on a lactate-containing medium. Thus in the opinion of previous and subsequent workers in the field, the patent is of questionable value to the petroleum industry.

Updegraff and Wren, (1954), seem to be of the opinion that when petroleum is used as a substrate for anaerobic growth, the utilization of certain non-hydrocarbon components might be the means of growth for sulfate reducers. This would depend greatly on the nature of the crude oil. The exact nature of such "non-hydrocarbon components" is certainly, at this point, still conjecture.

Updegraff (1954), carried out exhaustive studies in an attempt to determine the ability of sulfate reducers to use petroleum hydrocarbons as substrates for energy and growth. Of all the isolates they had at their disposal, they chose eleven for experimental work. Like other workers before them, they used hydrogen sulfide generation as evidence of bacterial activity, and not utilization of the hydrocarbons added. The results indicated that 4 of 11 strains of sulfate reducers were able to generate H_2S . This was perhaps some indication of hydrocarbon utilization.

Chemical measurements of free energies of reaction tend to cast doubt on the abilities of certain hydrocarbons to act as substrates for growth by sulfate reducers. Sorokin (1957), reported with very convincing chemical evidence that growth and sulfate reducing tests with methane and sulfate reducing bacteria were negative, while tests with hydrogen gas and carbonate yielded positive results. Thus he concluded that while the calculated free energy for the reduction of the sulfate ion by H_2 (~ 29 Kcal./mole) is sufficient for synthesis of cell material, reduction of sulfate by methane does not yield sufficient free energy for methane utilization and/ or hydrogen sulfide generation.

IV. Role of Denitrifying Microorganisms

Denitrifying organisms which have the ability to grow anaerobically were testing for their capacity to break down

hydrocarbons, (Hansen and Kallio, 1957). Most of their studies were carried out using the organism Pseudomonas stutzeri, which they isolated from soil. They were able to detect oxidation of dodecanoic acid, dodecanal, and dodecanol, anaerobically using nitrate as a terminal electron acceptor (oxidizing agent). Controls confirmed that the Pseudomonas was deriving all its nutritional and energy requirements from the above mentioned compounds. For most of their experiments, they used manometric measurements of nitrogen gas production as evidence of utilization of the hydrocarbons. However, in the presence of nitrate, under established anaerobic conditions, neither dodecane nor dodecene was oxidized. All the hydrocarbons tested were oxidized and utilized aerobically. Thus their work as well cast doubt on the importance of anaerobic oxidation of hydrocarbons. At the conclusion of their article they state, "There are probably very few anaerobic oxidations of paraffins (with the possible exception of methane) by either nitrate reducing or sulfate reducing bacteria."

V. Radioactive Uptake and Infra-Red Studies

It is interesting to note that Hansen, Kallio and others carefully avoided discussing methane in terms of anaerobic metabolism by sulfate reducers. The field research laboratories of the Socony Mobil Oil Company have, in the past, carried out various research projects con-

cerning the role of microorganisms in subsurface deposits. The results of a few of these projects have been reported in Davis, Petroleum Microbiology (1967). In one of these projects, the oxidation of hydrocarbons by Desulfovibrio desulfuricans was studied by means of radioactive labelling. In view of what was known about this topic from past research experience, it was decided that to seriously consider a relationship between these bacteria and hydrocarbon utilization, with a subsequent production of hydrogen sulfide, certain limitations would have to be allowed. Thus, under rather ideal experimental conditions, the oxidation of radioactive methane and ethane was tested. After incubation of their systems for approximately one month, both the carbon dioxide in the atmosphere above the plate cultures and the Desulfovibrio cells were radioactive indicating that both methane and ethane were utilized by the sulfate reducing bacteria.

This work is extremely significant in any discussion of microbiology pertaining to the field of petroleum research, since it is the first sound evidence to indicate that sulfate reducers play at least a small role in subsurface metabolism of petroleum components. Earlier work by Rosenfeld (1947), Uspenskii et al. (1947), Shturm (1951), and Dostalek et al. (1957), has also attempted to show positive utilization of petroleum hydrocarbons by sulfate reducing bacteria. Typically, their methods depended upon

the reduction of methylene blue as evidence of oxidation of hydrocarbons. Such methods are known to be insensitive and subject to errors because of many uncontrolled variables. Thus their work cannot be accepted with as much confidence as can the work carried out by the field research labs of Socony Mobil Oil Company. However, the work of Uspenskii et al. (1947), does show one interesting fact; namely that amounts of apparent hydrocarbon oxidation by sulfate reducers were extremely dependent on the type of petroleum used. Generally oils of high paraffinic character were preferred substrates as opposed to oils of high naphthenic or asphaltic character.

One last piece of evidence indicating anaerobic oxidation of paraffinic hydrocarbons by microorganisms, comes from work carried out by Chouteau et al. (1962). Using washed cells of Pseudomonas aeruginosa, they were able to show the dehydrogenation of n-heptane to n-heptene-1 anaerobically. Evidence for the formation of n-heptene-1 came from characteristic absorption by the double bond when samples were scanned by I.R. spectrophotometry. Their work did not however show any further utilization and/or oxidation of the hydrocarbons, nor was there any evidence of cellular growth.

VI. Summary of Anaerobic Influence in Formations

The role and importance of anaerobic microorganisms

within natural oil deposits is still in question. Their presence cannot be denied nor can their influence in terms of biogenic sulfur deposits, breakdown of oxidized organic materials, etc. What does become obvious is that it is exceedingly important to know whether anaerobes can in fact oxidize and utilize fully reduced hydrocarbons. If anaerobes do have this capability, then petroleum deposits cannot be thought of as static quantities of hydrocarbons; but must be thought of as bodies of petroliferous material still a dynamic part of the carbon cycle. This hypothesis is of academic interest to the microbiologist since it represents an additional influence of microorganisms on man's resources, but it is exceedingly distressing to the petroleum industry for two reasons. Firstly, it becomes virtually impossible for petroleums to be genetically related, since completely unpredictable changes could then occur to different petroleum deposits. This would cause them to be ranked as being genetically different when in fact they may once have been genetically related. A second and perhaps more compelling reason for concern is the fact that if metabolism of oils within the deposits can occur, the resulting oil would most likely be of inferior refining quality. Since such a process, if it occurs, would take place over a very long period of time, deposits found to be degraded would have to be considered for all practical purposes to have been permanently ruined.

VII. Evidence for Formation Oil Degradation

In reviewing the literature, there are a few articles and papers which do deal with the subject of petroleum degradation within untapped oil reserves. Perhaps the best review of the subject comes from a paper presented at the Symposium on Petroleum Transformations in Geologic Environments during the American Chemical Society meeting of September, (1969). The authors of the paper, J. C. Winters, and J. A. Williams cautiously suggest that microbial action may be responsible for certain changes observed within formation oils of the Powder River Basin field in Montana. They first make clear that crude oil may be altered in the reservoir by several natural phenomena. Oils maturing in very deep formations can be changed to such an extent that they no longer resemble any of their genetically related counterparts still deposited at more shallow depths. Oils in shallow formations near outcrops show evidence of oxidation either from actual oxygen penetration of the formation or by dissolved oxygen within the groundwater coming into contact with the oil.

A more unusual type of alteration is occasionally found where certain crude oils have lost portions of their paraffin fraction leaving them with what is termed "naphthenic character." Certain geochemists have speculated that microorganisms have selectively destroyed these paraffins. Winters and Williams attempt to show that such

a speculation has merit for two reasons. The first reason is that aerobic bacteria are known which selectively oxidize n-paraffins, and the second and more acceptable reason is that the altered crudes are usually found in relatively shallow reservoirs, which would be more easily reached by percolating surface waters bearing oxygen necessary to sustain microbial oxidation processes.

VIII. Implications of an Aerobic Process in Formation Oil Degradation

It is rather obvious that Winters and Williams as well as other collaborating geochemists are of the opinion that an anaerobic degradative process is not important here but rather an aerobic process, mediated by dissolved oxygen within groundwater. If this is true and since actual detectable amounts of dissolved oxygen have been found in percolating groundwater, then any evidence of viable aerobic or microaerophilic organisms within this groundwater would certainly lend favor to a theory implicating aerobic microbiological degradation of certain shallow oil deposits.

The paper by Williams and Winters gives reference to more direct evidence of microorganisms in the Bell Creek field within the Powder River Basin area. By using portions of water co-produced with some of the oil samples as culturing medium, aerobic microorganisms were in fact isolated. Some of these organisms were found to have the

capacity to metabolize both n-butane and n-hexadecane at reservoir temperatures (95-105°F), while some did not exhibit this ability. No further mention was made in the paper as to whether these organisms were maintained for further experimentation, whether they were classified taxonomically, nor if they were submitted to any type culture collections to make them available to microbiologists elsewhere.

IX. Specific Evidence of Petroleum Degradation

The data presented by Winters and Williams indicated that in nearly every case, isolation of microorganisms from groundwater samples correlated with degraded oils within the same area; and areas where no apparent oil degradation had occurred did not yield any microbial isolates from the groundwaters.

Wenger and Reid (1958) who earlier carried out analysis of the Powder River oils from Montana were able to show that altered or degraded oils contained up to four to five times as much nitrogen as unaltered oils from the same producing formation. At that time the observation was left unexplained. Winters and Williams have since chosen to believe that this increased nitrogen content of the altered oil must be a product of microbiological activity, possibly amino acids derived from the accumulated microbial mass.

In studying gas chromatograms of saturate samples from throughout the Bell Creek field, on the Montana-Wyoming

border, examples of degraded oils always showed portions or all of the n-paraffins as being lost, leaving only an isoparaffin envelope. Also, many of the chromatograms showed isoprenoid enrichment in the case of degraded oils. This was used as further circumstantial evidence of bacterial attack since it is known that isoprenoids are often very refractive to bacterial breakdown.

The Bell Creek field has received the most investigation of any oil field in terms of explaining oil degradation via a microbial process. However, other fields have shown similar evidence of petroleum degradation. The east and west Hackberry fields in Cameron Parish, Louisiana have produced oils showing evidence of n-paraffin loss as well as some loss of singly branched isoparaffins. It is suspected that microorganisms began to consume the more difficult branched paraffins after the n-paraffins had been destroyed.

A wildcat well in North Africa has produced petroleum showing extensive n-paraffin loss when compared to a genetically related oil. The formation temperatures of this well were shown to be within tolerance limits for bacterial growth.

As mentioned in the introduction, Imperial Oil Limited has also found evidence of oil degradation in a deposit in Saskatchewan which they now suspect was microbiologically mediated. Thus far, attempts to isolate microorganisms

from groundwater samples within the oil deposits have not been carried out either in the Saskatchewan case nor in the case of the North African wildcat well.

As a rough estimate from analyses carried out in the American Oil Company labs and the Pan American Petroleum Corporation labs, approximately 10% of all crude oils characterized have compositional characteristics strongly suggesting microbial alteration. Since almost every major oil field in the world contains at least a few apparent examples of altered oils, it would seem that much investigation is needed to isolate and characterize microorganisms found in these deposits. Unfortunately on this continent, very little work has been carried out toward this end except on the Bell Creek field in the Powder River deposit.

X. Oil-Brine Isolations in Japan

The literature in general shows few attempts to isolate bacteria from groundwater or co-produced waters from oil wells. Much of what has been done has either remained unpublished or has been published in journals not widely circulated. However, research in Japan on this specific topic has carried on at an ever increasing rate and much of the work has been published. Their chief interests lie in the production of inexpensive single cell protein from various industrial fractions of petroleum. To generate a series of bacteria and yeasts which would utilize such

substrates, it was decided to investigate natural oil deposits in an attempt to find organisms with these capabilities. Thus a series of publications were made by two microbiologists, describing the results of these attempts.

Work in Japan concerning hydrocarbon utilization has in fact been in progress since Miyoshi first revealed the decomposition of paraffin wax by Botrytis cinera, (1895).

Iizuka and Komagata (1964a) carried out taxonomic investigations on liquid paraffin-utilizing bacteria isolated from oil brines and other related materials collected in 1956 from oil fields in Japan. Isolation was carried out by enrichment culture. Kerosene and crude oil were used as a sole source of carbon, and oil brine, flooding-water, cutting pieces, drilling mud, bottom waters of oil tanks, etc. were chosen as starting materials for enrichment. After several weeks, numerous hydrocarbon utilizing bacteria had been isolated. They were identified as being of the following species: Corynebacterium hydrocarboclastus, Pseudomonas nitroreducens, Pseudomonas maltophila, Brevibacterium lipolyticum, Pseudomonas desmolytica, Flavobacterium ferrugineum, and Alcaligenes faecalis. It is rather interesting to note that no sulfate reducers were isolated or mentioned, although their method may have precluded such possibilities. Their evidence lends further credence to the proposal that areobes or microaerophiles are active in certain

oil deposits.

Earlier, Iizuka and Komagata (1961) carried out isolation experiments using only oil-brines as starting materials. Apparently no identification of the organisms was carried out. It was stated that the oil brines taken from 200 to 700 meter depths contained about 10^7 aerobic bacteria and about 10^4 anaerobes/ml. This indicates a very high level of metabolic activity within or around the deposit.

Iizuka and Komagata (1964a) carried out isolation procedures specifically for pseudomonads using oil brines obtained in Yabase, Higashiyama, and Nishiyama petroleum fields in Japan. Sampling of the oil brines was made at oil storage tanks and direct sampling from oil layers was undertaken, using bottomhole samplers. After much work with differential media, the following species were identified: Pseudomonas iners, Pseudomonas stutzeri, Pseudomonas putrefaciens, Pseudomonas azotoformans, Pseudomonas nitroreducens, and Pseudomonas aeruginosa. Since some of these organisms, at least, are facultative, Iizuka and Komagata are of the opinion that pseudomonads play an important role as the main members of microflora of petroleum zones.

The third paper in a series concerning microbiological studies on petroleum and natural gas by Iizuka and Komagata (1965a), involved the determination of: Brevibacterium, Arthrobacter, Micrococcus, Sarcina, Alcaligenes, and

Achromobacter. Examples of each were found in the oil brine samples, but only Arthrobacter, some Micrococcaceae, and Sarcina showed any degree of growth at 37°C. Thus the metabolic activity of the others must be severely hampered under oil formation conditions.

Izuka and Komagata have also succeeded in isolating aerobic or facultative organisms in the Higashiyama oil mining field (1964b), from the Yabase and Nishiyama oil fields (1965c), and the Niigata and Mobara gas fields (1965d). In short, these two workers have isolated aerobic and anaerobic, viable microorganisms from practically every economically important oil deposit in the Japanese islands. Though the geology, the deposit temperatures and the deposit depths of the Japanese oil fields differ from those in North America and elsewhere, it still proves that the potential for actively metabolizing microorganisms within oil deposits does exist. What is surprising about the papers by Izuka and Komagata is the complete absence of any mention of sulfate or sulfite reducers. Their use of peptone medium and meat extract medium in test tubes during the early isolation attempts, would certainly have shown FeS accumulation had the sulfate or sulfite reducers been present. Since not all their methods could preclude the enrichment of sulfate reducers, one must conclude that either Japanese oil fields contain only low numbers of such organisms, or their presence among the isolates was simply

not mentioned. It would seem, however, that all such experiments, whether carried out in Japan, North America, or elsewhere would most likely yield isolates of both the sulfur reducing group, and the large non-sulfur-reducing group, i.e. the aerobic and facultative organisms.

MATERIALS AND METHODS

I. Sources of Isolates

The soils from which microorganisms were isolated for this investigation came from three sources. The first set was taken, in June 1970, from a disturbed Grey-Wooded soil beneath a christmas tree* site at one of Imperial Oil's producing wells, in the Judy Creek field. The sample had originally been Grey-Wooded but was mostly composed of parent material as a result of grading operations and had been dosed periodically with crude oil for almost ten years. The second soil source was a greenhouse clay loam which had been treated with petroleum, more than a year prior to sampling in April 1970. The third soil sample was taken five miles northwest of Salmon Arm, B. C., on December 29, 1970, at a site of continuous diesel fuel spillage. This soil was used specifically for isolation of possible psycrophilic petroleum degraders; as soils in the Salmon Arm area remain at 2 - 3° during the winter months.

II. Cultural Conditions

All bacterial isolates were obtained from the soil samples described in Materials and Methods (I).

For all the isolation procedures, Butlins medium,

*A term used in the oil industry to describe a device mounted on a well head, possessing numerous delivery pipes and valves; its appearance distantly reminding one of a christmas tree.

(Butlin, 1949), was used as the basic growth medium. Its composition is as follows:

K_2HPO_4	0.5 g
NH_4Cl	1.0 g
Na_2SO_4	2.0 g
$CaCl_2 \cdot 6H_2O$	0.001 g
$MgSO_4 \cdot 7H_2O$	1.0 g
Sodium lactate (60%)	2.5 mls
Difco Yeast Extract	1.0 g
$FeSO_4$	trace
Distilled-deionized water	1,000 mls

pH after autoclaving: 7.3

Although this medium was originally designed for isolation and growth of sulfate reducing bacteria, it proved to be suitable for growth of microorganisms with hydrocarbon-utilizing abilities.

Additional isolates also were obtained by using two modifications of Butlins medium. The first variation involved the addition of KNO_3 (2.0 g/l.), for purposes of enriching denitrifiers, and was termed the B+N medium. A carbon free medium was obtained by removing the yeast extract and sodium lactate from the NO_3^- -enriched medium, yielding a basal salts solution, termed B+N salts.

III. Procedure for Isolation of Cultures

Because of the reasons presented in the introduction, the few anaerobic isolates obtained during enrichment procedures were not used to any great extent in the investigation. The main work involved only the aerobic or facultative isolates.

As an initial isolation procedure, 18 x 150 mm tubes, containing Butlins medium and B+N medium were inoculated in quadruplicate with 0.1 g samples of the soils listed in Materials and Methods I. In addition, the soils were plated on Butlins agar (Butlins medium + 1.5% Difco agar), and B+N agar; each plate being spread with 0.1 mls of a 10% suspension of each of the soils. Tubes were incubated under aerobic conditions; while the plates were incubated in separate, freshly charged BBL anaerobic jars, both at 30°. Growth and denitrification were apparent in the tubes and plates after five days incubation, when all tubes showing growth and all isolated colonies were re-streaked on Butlins or B+N agar, in duplicate. Half of the plates were incubated anaerobically at 30°; and the remaining plates kept at 30° under aerobic conditions. The great diversity of isolated types, both aerobic and anaerobic, were re-streaked several times on their respective agars in an attempt to obtain pure isolates. At the same time, cultures were maintained in liquid medium in an attempt to maintain the original strains. In addition, isolates

were maintained on 1% B+N sloppy agar at 3°.

The technique of soil enrichment or elective culture, devised by Winogradsky and Beijerinck and described by Bullock (1960), was also used to generate mixed cultures of hydrocarbon utilizing bacteria. In this investigation, enrichments were incubated at mesophilic and psychrophilic temperatures. Three, two liter flasks containing one liter of B+N salts medium + 1, 5, or 10 mls of North Cantal oil respectively, were inoculated with christmas tree site soil and incubated at 30°. Flasks were shaken on a New Brunswick rotary shaker at 300 r.p.m. (one inch eccentricity). In addition, two, two liter flasks containing one liter of B+N salts medium + 1 ml of North Cantal oil/flask, were inoculated with christmas tree site and Salmon Arm soil. These flasks were incubated at 4°, at 280 r.p.m. (one inch eccentricity). Inoculations for all flasks consisted of 10 mls of a 10% soil suspension.

In the case of the mesophilic enrichments, viable count progressions were obtained throughout the isolation procedure. Six successive transfers to fresh B+N salts + 1 ml North Cantal oil/liter were made, at 7 day intervals, starting with inoculum from the original enrichment flasks dosed with 1 ml of North Cantal oil. The initial enrichment flasks dosed with 5 and 10 mls of North Cantal oil were not transferred since the bacterial populations enriched in all three mesophilic flasks were morphologically

similar. At the end of the seventh transfer, a stable bacterial mixture (in terms of colony type), had been developed with petroleum-utilizing capabilities.

In the case of the two psychrophilic enrichments, (christmas tree and Salmon Arm), successive transfers to fresh B+N salts + 0.1% North Cantal oil were made at 14 day intervals. Viable count estimations were begun after the fifth transfer, when it was certain, on the basis of macroscopic and microscopic observation, that a stable bacterial population had developed.

Both the mesophilic and the psychrophilic mixtures have maintained their hydrocarbon-utilizing abilities for at least six months of regular transferring on a B+N salts medium containing 0.1% North Cantal oil.

IV. Sources of Chemicals, Reagents, Solvents, and Crude Oil Samples

All chemicals and reagents used were of reagent grade and obtained from commercial sources. The solvents used for chromatographic studies were of infra-red spectranalyzed quality, and were obtained from Fisher Scientific Company. Silica gel (28 - 200 mesh) and F-20 Alcoa Alumina gel (80 - 200 mesh), which were used in liquid chromatographic analyses described later in the text, were purchased from Matheson, Coleman and Bell. Gas chromatographic columns, containing Chromosorb P as the solid support and OV-1 reagent as the liquid phase, were purchased from Varian Aerograph in the

form of pre-packed columns. The reagent, (OV-1), was also obtained from Imperial Oil Research Laboratories, Calgary, Alberta, as a pure phase coated to Chromasorb P. This was used for the custom-making of a column specifically for this project.

The four petroleum samples used in this investigation were obtained by Imperial Oil Co. from various areas of the Weyburn oil field in the province of Saskatchewan. The analyses of these oils are presented in Table I in the order of increasing probability of being previously microbially altered. Laboratory samples of these oils were stored in sterile one liter glass bottles at 2°.

V. General Experimental Culturing Procedures

For most experiments described later in the text, the following general procedure was adhered to. Culture flasks were of the two liter Erlenmyer type, unless stated otherwise. Excluding the aeration experiments, described later, the flasks were filled with one liter of either Butlins medium, B+N medium, or B+N salts medium. These were routinely autoclaved for 20 minutes at 121°. Inoculation of the flasks, with few exceptions, was with liquid culture grown on the same medium plus varying amounts of North Cantal oil. After inoculation, specific amounts of North Cantal oil were added to each flask, the amount depending on the experiment. Flasks containing medium, inoculum, and petroleum (the latter being absent in control flasks) were

TABLE I
LIQUID CHROMATOGRAPHIC ANALYSES OF OILS
FROM THE S. E. SASKATCHEWAN FIELDS

Oil	Weight %				
	Sulfur	Asphaltenes	Sats.	Aromatics	NSO's
North Cantal	0.97	5.1	5.3	34.8	6.2
Workman	1.40	8.4	52.0	36.0	5.0
Stoughton	2.22	15.7	36.0	38.0	9.6
Lost Horsehill	2.56	11.0	35.0	42.2	12.0

All values given here are as weight percentages of each oil; the oils having previously been topped at 36.5° for 19 hours to stabilize their initial weights.

Sulfur analyses result from the sulfur to be found in organic and inorganic forms within the petroleum samples.

All other listed components are described in the Materials and Methods VIII section.

then incubated at either 30° or 4°, depending on whether mesophilic or psychrophilic conditions were required. Shaking, for purposes of aeration, was carried out using New Brunswick shakers (eccentricity in all cases, 1.0 inches).

In the case of the mesophilic aeration experiments undertaken in the investigation, the above method was followed. The only changes involved the volumes of B+N salts, where varying volumes from 250 mls to 1,000 mls/ 2 liter flask were used.

For experiments involving utilization of purified petroleum components, 200 ml volumes of B+N salts were used in 500 ml Erlenmyer flasks. Otherwise conditions were as stated earlier.

For the only experiment in the investigation, involving growth in test tubes, the following method was devised. Fifteen ml volumes of Butlins medium, B+N medium, etc. were placed in 18 X 150 mm tubes; and after capping, all tubes were autoclaved for 15 minutes at 121°. One ml of North Cantal oil was aseptically dispensed to each tube. Inoculation by sterile loop, from the isolates maintained on 1% B+N agar, was the final step before incubation at 30°. Evidence of petroleum emulsification was obtained, after the incubation period, by shaking each tube and a control tube simultaneously. If emulsification had occurred, the petroleum would remain in suspension much longer, in the

test system, than in the control tube.

VI. Estimation of Bacterial Growth

Bacterial growth was followed by the plate count procedure using the spread plate method. Ten-fold serial dilutions of samples taken from growth flasks were made by transferring 1 ml aliquots to 9 ml dilution tubes. Dilutions were continued to 10^{-7} . The dilution medium was 3 mM phosphate buffer, pH 7.3. One-tenth ml samples of the dilutions were plated in triplicate on B+N agar. For mesophilic experiment, plates were incubated for 3 to 5 days at 30°; while for all psychrophilic experiments, plates were incubated for 10 days at 4°.

VII. Recovery of Residual Oil from Cultures

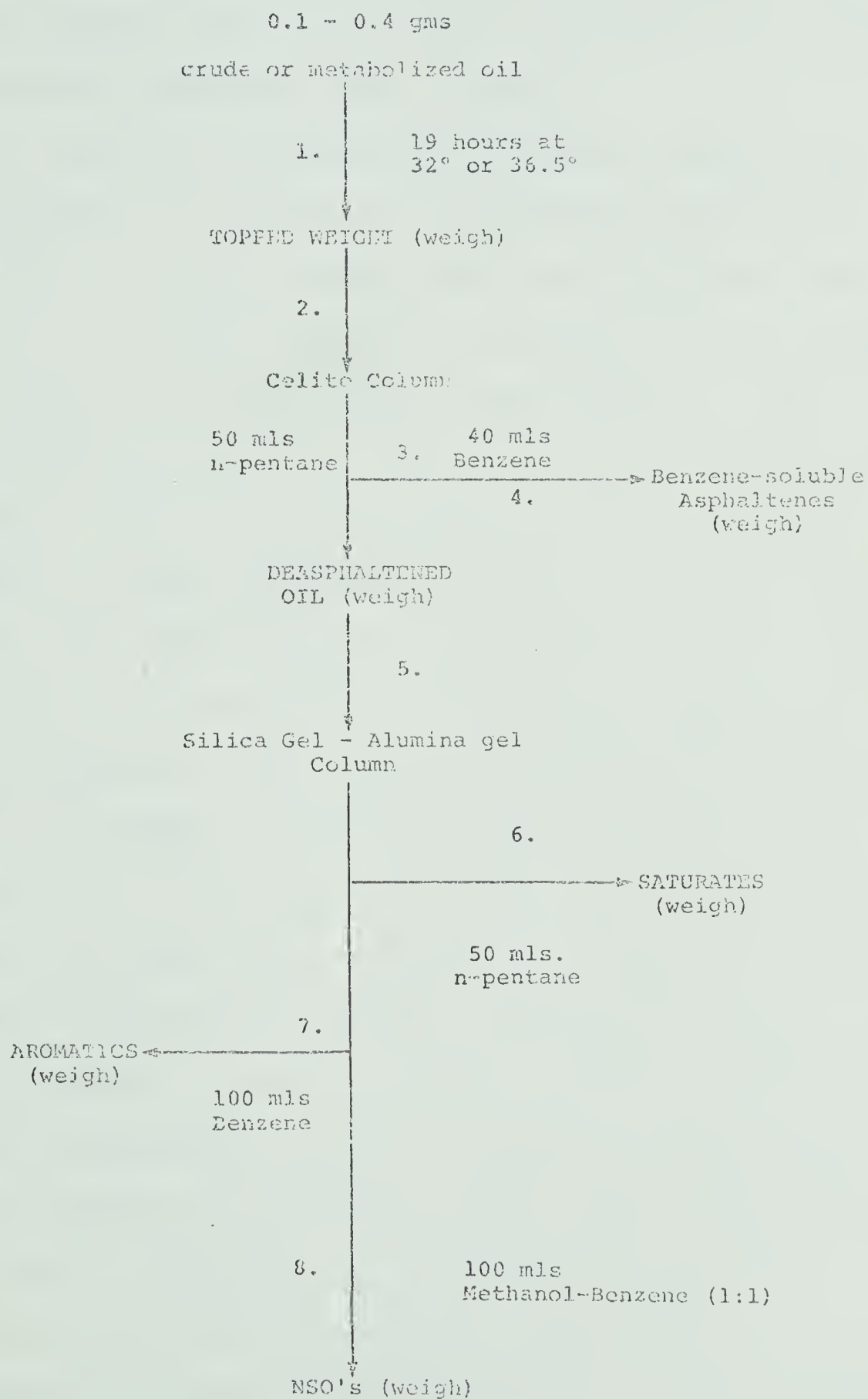
In order to recover residual petroleum from culture flasks, for purposes of analyses, a n-pentane extraction procedure was devised. Liquid cultures containing residual petroleum were split into 500 ml amounts and each fraction extracted three times with a 50 ml aliquot of n-pentane, (total volume = 150 mls). The aqueous phase containing cells was drawn off, each time, to the interfacial area containing liquid B+N salts, asphaltenes, and n-pentane. This interfacial area and the remainder of the n-pentane was then drawn off and placed in a beaker. When all portions of a culture had been extracted, all the pentane washes and interfacial samples were pooled and evaporated

to dryness in a fumehood. The balance of the liquid B+N salts plus cells was discarded. Because of the residual aqueous volumes brought over in the interfacial samples, it took 5 to 6 days to completely dry the extracted oil. What remained was a crust at the bottom of the beaker composed of residual oil and organic material which appeared to be insoluble in polar and nonpolar solvents at room temperature. The residual petroleum was then easily recovered from the beaker by adding 10-20 mls of benzene. This was allowed to stand for thirty minutes. Then, using a Pasteur pipette, the sides of the beaker were thoroughly washed with the same benzene. The petroleum was now a homogenous solution in the benzene and an aliquot was removed using a propipette. If care was exercised, the residual organic crust at the bottom of the beaker was never disturbed and a clean, pure residual oil sample was obtained.

VIII. Liquid Chromatographic Fractionation of the Petroleum Samples

Samples of crude petroleum or metabolized petroleum were subjected to fractionation in the following manner. Amounts of the material ranging from 0.1 to 0.4 grams were first of all carried through a topping process. In this procedure, the petroleum was placed in a tared beaker and kept at either 32° or 36.5° (stipulated in Results and Discussion), under forced draft conditions for 19 hours.

TABLE II
SCHEMATIC FLOW SHEET FOR LIQUID CHROMATOGRAPHIC
SEPARATION OF PETROLEUM FRACTIONS



The resulting weight of petroleum was termed, "topped weight of oil." By this method, the light aromatics and all non-aromatic hydrocarbons up to nC_{15} were volatilized off, leaving a weight which did not vary throughout the course of subsequent chromatographic analyses.

The liquid chromatographic procedure used in the investigation was similar to that used by Imperial Oil Company Ltd. In this method, the benzene-soluble asphaltene fraction of oils was recovered by passing the oil through a 1 X 15 cm bed of Hyflo Super - Cel (Fisher). The deasphalted oil obtained from this step was weighed and then further fractionated by adsorption chromatography. In this step, a column was packed with 10 ± 0.2 gms of activated 29 - 200 mesh silica gel (Matheson), and then with 10 ± 0.2 gms of activated F-20 alumina gel (Matheson), both in n-pentane. The resulting dual phase gel bed was 1 X 40 cm in height. The deasphalted oil was layered onto the top of the column and eluted sequentially with n-pentane, benzene, and 1:1 benzene-methanol, thus separating the sample into three fractions. Therefore by using a two column procedure, a petroleum sample could be fractionated into four components, the benzene-soluble asphaltenes, the saturate fraction, the aromatic group, and a final fraction termed the NSO component. In addition, by summation of the weight differences of components from the celite column, the weight of benzene-insoluble asphaltenes can be calcu-

lated. By component summation in the case of the alumina gel-silica gel column, the weight of insoluble NSO component plus overall loss can be calculated.

The asphaltenic fraction of petroleum is composed of a complex, ill-defined mixture of fused aromatic rings and fused, saturated cyclic compounds which may or may not have groups substituted on the various rings. As a group, the asphaltenes are not soluble in n-pentane, and exhibit partial solubility in benzene. That portion of the asphaltenes not exhibiting benzene solubility on passage through a celite column is termed the benzene-insoluble fraction.

The saturate fraction is composed of normal straight chain alkanes whose length, in the topped oils of this investigation, range from nC_{15} to nC_{30} or nC_{34} , as well as branched-chain alkanes and cyclic alkanes. In addition, this fraction may or may not contain the isoprenoids, e.g. pristane and phytane. The saturate component of an oil is soluble in both n-pentane and benzene.

The aromatic group of hydrocarbons comprises a wide variety of substituted aromatic rings as well as groups of two, three, four, and five fused aromatic rings with substituted groups. There may be one to five substituted methyl groups in any of a number of combinations on a ring or rings. A few aromatic groups show hydroxyl-substitution, but these are in very low amounts. As a group,

the aromatics are soluble in benzene and n-pentane.

The last group of hydrocarbons to be eluted from a silica gel-alumina gel column with a 1:1 mixture of benzene-methanol, the NSO fraction, is unfortunately another ill-defined component of petroleum. Basically, the NSO fraction is an arbitrary fraction eluted from the silica gel-alumina gel column on the basis of its molecular weight and solubility in 1:1 benzene-methanol. Though it is partly composed of components containing nitrogen, sulfur, or oxygen, or any combination of all three which do lend an increased degree of polarity, the aromatic fraction is also known to contain some of the same inorganic constituents as does the asphaltenic fraction. Thus the molecular weight, and solubility of the NSO fraction are the only properties which actually distinguish it from the aromatic fraction. As a group, they are soluble in benzene and partially soluble in methanol as well as n-pentane.

IX. Gas Chromatographic Analysis of Saturate Hydrocarbons

Gas chromatographic analysis of saturate fractions was accomplished using a Varian Aerograph, Model 1740-1 equipped with a flame ionization detector for enhanced sensitivity.

All carrier gasses used were purchased from commercial sources, and were purified by passage through Hydro-Purge Molecular Sieve 5A filters before being passed through the column or detector.

In the initial stages of the project, various columns were tested for their abilities in giving good chromatographic

separation, minimal column bleed, and long term stability. Five percent SE-30 columns were initially used; however their lack of complete resolution of all peaks, as well as problems of excessive noise and bleed, made them undesirable for temperature programs exceeding 300°. The second system used was a matched set of 1/8" I.D. glass columns containing 5% OV-1 on Chromosorb P. Although, initially, resolution was excellent, excessive bleed problems as well as eventual splitting of the phase within the columns, ruled them out as columns of choice at this time. Eventually a column for the project was made according to specifications set forth by Imperial Oil at their research facilities in Calgary, Alberta. This column was constructed from 1/16" I.D. stainless steel and was 10' in length. It was packed, using vacuum and vibration, with Chromosorb P (100-120 mesh), coated with 3% OV-1 (liquid phase) and conditioned at 325° for 72 hours before it was used for analytical purposes. Low bleed rates plus reasonable sensitivity made this an exceedingly useful column, as compared with the larger volume OV-1 columns used previously. The following settings were used for these analyses:

Linear temperature program: 50 - 325°.

Rate of programming: 10°/minute.

Injection block temperature: 300°.

Nitrogen flow rate: 12.35 mls/minute.

Hydrogen flow rate: 15.0 mls/minute.

Air flow rate: 300 mls/minute

Recorder chart speed:

(a) Varian Model 20: 0.5"/minute.

(b) Beckman: 0.33"/minute.

Range: 10^{-10} amps./millivolt.

Attenuation: X 8.

Under the above conditions, injections of saturates at a concentration of 40 micrograms/microliter of solvent gave optimum response and clear resolution of n-saturate components. For all saturate analyses in this project, benzene was used as solvent, since volatilization problems were not as severe as with n-pentane, and benzene passed through the OV-1 column as a distinct peak well in advance of all other components in the sample.

Injection of saturate samples on to the OV-1 column was accomplished with a 5.0 micro-liter syringe (Precision Sampling Co.). Volumes injected varied according to the amount needed to ensure 40 micrograms of saturate sample entering the column.

Characteristically, chromatograms from this column possessed an S-shaped profile. The envelope present in all these chromatograms was a combination of isoparaffins and cyclic alkanes not resolved under these conditions, as well as a small but constant amount of column bleed which occurred at temperatures in excess of 100°.

RESULTS AND DISCUSSION

I. Initial Testing of Isolates

All isolates obtained from the plating procedures were screened to determine their action on petroleum. The growth characteristics of these isolates were studied using liquid Butlins and liquid B+N medium containing 1 ml of North Cantal oil. The complete test series was carried out in 18 X 150 mm tubes as described in Materials and Methods V. All incubations were for 7 day periods at 30°. The major visual effect of microbial activity which was scored was the emulsification of the oil. The nature of the emulsifying process was not investigated, but it may well have been due to a natural detergent agent as described by Holdom and Turner (1968).

Thirty-six isolates emulsified oil on both Butlin's medium and B+N medium; while 32 isolates emulsified oil only on B+N medium. Twelve isolates emulsified oil only on Butlin's medium. Therefore, a total of 80 isolates demonstrated the ability to emulsify oil on Butlin's, B+N, or both.

Of the 195 isolates originally obtained, 28 were denitrifiers and 20 of these were also capable of emulsifying oil.

Except for the sulfate reducing isolates, which were subsequently discarded from this investigation, only the 28 denitrifying organisms had the ability to grow in liquid

B+N medium under the anaerobic conditions of a BBL anaerobic jar.

As mentioned previously, the sulfate reducing isolates were not used in the course of this investigation. When growing on Butlin's medium + North Cantal oil, these sulfate reducers exhibited only slight emulsifying capabilities. In addition, after extensive testing it was found that these sulfate reducers produced poor growth on a salts medium with crude oil as a sole carbon substrate. Lastly, crude petroleum, after extensive exposure to the sulfate reducers, did not show any significant chromatographic differences, indicative of bacterial attack. For these reasons, work with the sulfate reducers was carried no further. However, the fact that the sulfate reducers were isolated in great numbers from the christmas tree soil is significant. It would seem that either the constant spillage of oil on the site caused sulfate reducing bacteria to be stimulated as a zymogenous population, or that the oil contained the organisms already in numbers sufficient to inoculate the soil. No further studies were made to clarify this point.

II. The Examination of Growth of a Pseudomonas Isolate (6c(b)100) under Aerobic and Anaerobic Conditions on North Cantal Oil

As mentioned in the introduction, many workers have, in the past, attempted to resolve the question of the

ability of Pseudomonas strains as well as sulfate reducers to grow anaerobically with petroleum as a sole carbon substrate. In this investigation, an experiment was designed to determine whether a denitrifier could utilize crude oil as a partial or complete carbon and energy source, aerobically and anaerobically.

An isolate, 6c(b)100, showing visible emulsification abilities on North Cantal oil was identified as being of the genus Pseudomonas. An overnight culture of this isolate produced large amounts of a blue-green pigment when grown on B + N medium; and when streaked on a plate count agar slant the isolate showed heavy yellow growth. A Gram stain of the culture showed Gm (-) rods singly and in pairs. It possessed denitrifying capabilities and produced anaerobic, acidic (yellow) reaction on Hugh and Leifson medium. A Tromsdorf's test showed NO_2^- formation and Kovak's reagent produced blackening indicative of positive oxidation of N,N' dimethyl-phenyldiamine.

In this experiment, an increase in viable counts over a control was used as being indicative of petroleum utilization. The design of the experiment was as follows. All flasks were of 500 ml size and contained 200 mls of medium. Eight such flasks were prepared in the order shown in Table III. The first four flasks were shaken aerobically at 300 rpm (eccentricity, one inch) at 30°. The latter four flasks were placed in BBL anaerobic jars at 30° and

the cultures were agitated with magnetic stirrers. All oil charges were with North Cantal oil at the rate of 2 mls per flask, and were made at the time of inoculation.

Inoculation was from inoculum grown overnight on B+N medium at 30°. A control, as defined in this experiment, was any flask not charged with oil. Viable counts were carried out as described in Materials and Methods VI. Results of the plate counts are presented in Table III.

It would seem, that under aerobic conditions, and with an alternate source of carbon, additions of petroleum yielded no stimulation of growth. There was, however, plenty of evidence of visible oil emulsion by the bacterial growth. An increase in numbers was achieved on B+N salts medium, aerobically, when petroleum was the sole carbon substrate. Anaerobically, petroleum produced no significant growth stimulus when provided as either an alternate or sole carbon substrate. Therefore, this seems another compelling piece of evidence indicating the inability of pseudomonads to utilize petroleum anaerobically.

III. Growth of Pseudomonas Isolate 6c(b)100 on Petroleum for Chromatographic Analysis

The experiment described in Results and Discussion II attempted to show the ability or inability of isolate 6c(b)100 to utilize North Cantal oil aerobically and anaerobically, by measuring increase in viable counts. This certainly did yield some indication of petroleum

TABLE III
 VIABLE COUNTS OF PSEUDOMONAS SP.

Growth Medium and Environment	*72 Hour Viable Counts/ml
B+N + oil (aerobic)	1.0×10^9
B+N control (aerobic)	1.1×10^9
B+N salts + oil (aerobic)	3.8×10^8
B+N salts control (aerobic)	4.8×10^7
B+N control (anaerobic)	6.8×10^8
B+N + oil (anaerobic)	7.8×10^8
B+N salts control (anaerobic)	4.4×10^7
B+N salts + oil (anaerobic)	5.8×10^7

*All flasks inoculated to an initial viable count of
 3.8×10^7 cells/ml.

utilization, and therefore degradation, at least under aerobic conditions. However the experimental design was weak in the sense that no indication could be gained as to which component(s) of the petroleum might be undergoing degradation. Therefore it was decided that a major scale experiment be carried out on a sufficiently large quantity of oil to determine, via chromatographic means, what changes were being rendered aerobically and anaerobically.

Twenty-four two liter flasks, each containing 1.0 liters of B+N medium were prepared, autoclaved at 121° for 20 minutes, and cooled. Each was then inoculated with 10 mls of an overnight culture of the organism. They were shaken at 300 rpm at an incubation temperature of 30° for 18 hours. At this point, the cells were harvested by centrifugation yielding a total wet weight of cells of 16 grams. The cells were resuspended in 0.05 M phosphate buffer, pH 7.2, and washed in a total of 1500 mls of the same. The experiment was then set up using 2, 2 liter Buchner flasks (anaerobic test and control), and 2, 2 liter Erlenmeyer flasks (aerobic test and control). Each Buchner flask contained 1500 mls of B+N medium (sodium lactate removed for purposes of this experiment); while each Erlenmeyer flask received 1,000 mls of the same. After autoclaving, each test flask received 8 wet grams of cells. The aerobic test and control flasks were incubated at 30° with agitation of 300 rpm, immediately after the addition

of five mls of North Cantal oil to each flask. The anaerobic test and control Buchner flasks were incubated at 30° and provided with methylene blue indicators. The flasks were sparged with purified Linde N₂ gas at a rapid rate for 4 hours, when five mls of North Cantal oil were added by syringe to each flask. The oil-medium mixture was agitated with magnetic stirring bars.

The test anaerobic flask (inoculated), reduced the methylene blue after 24 hours incubation while the control flask (uninoculated), failed to show complete anaerobiosis until 72 hours of incubation time had elapsed. These results suggest the oil was being, in part, oxidized at the expense of methylene blue. All flasks were incubated for ten days when the residual petroleum was recovered by the floatation and centrifugation technique, (a method which later proved most unsuccessful as explained elsewhere in the text).

The petroleum samples were centrifuged in the presence of Na₂SO₄ for drying purposes. Samples of the residual, dried crude oil were sent to Imperial Oil Ltd., Calgary, where they were analyzed; as well, liquid chromatographic analyses of the North Cantal standard samples were carried out in our own lab. The percentage values obtained from the liquid chromatography are presented in Table IV. The values obtained for the standard samples form the basic standard analysis to which all other comparisons in this and subsequent experiments were made.

TABLE IV

LIQUID CHROMATOGRAPHIC ANALYSES OF RESIDUAL OIL
 SAMPLES AFTER METABOLISM BY PSEUDOMONAS SP.

Component	North Cantal	Aerobic	Anaerobic
	Standard	Experimental	Experimental
Weight %			
Benzene-soluble asphaltenes	2.5	16.65	4.8
Benzene-insoluble asphaltenes	6.8	29.6	8.8
Saturates	51.1	26.7	45.2
Aromatics	31.2	19.3	28.7
Soluble NSO	8.6	8.9	8.8
Insoluble NSO	0.0	0.9	4.1

Topping temperature for both experimental and standard samples: 36.5°. All values expressed as weight percentage of the topped weights used.

The apparent loss of saturates in the aerobic sample would make it appear that significant degradation of the petroleum had occurred. This observation is not so evident when one considers the saturate GLC profiles (Figure 1). On comparing ratios of n-saturate: isoparaffin heights between the control GLC profile, and that of the aerobic test sample, one cannot detect enough difference to justify a decrease in saturate content from 51% to 26%. However, the fact that the isoparaffin envelope encompasses more of the total profile area in the case of the aerobic test saturates indicates saturate utilization. Also the phytane peak, immediately following the nC_{18} peak is a minor component in the control profile, but is equal to the nC_{18} peak in the aerobic profile. This also suggests that the petroleum has been degraded aerobically at the expense of the saturate component. The rather dubious results obtained by liquid chromatography, especially in the aerobic case, are probably due to contamination by non-hydrocarbon components. This is evidenced by the disproportionate change in benzene-soluble asphaltenes which increase by a factor much greater than would be anticipated due to negative enrichment. This is assuming only saturates were being utilized. Therefore, it is clear that reclaiming degraded oil by flotation or centrifugation will not give a representative sample, as pointed out later. Neither the liquid nor the gas chromatographic analysis of the residual

oil from the anaerobic test showed any significant amounts of degradation occurring. It is due to this very lack of petroleum breakdown in the anaerobic culture that extracting of the oil by floatation was more successful than in the case of the aerobic culture where much more emulsification had occurred.

In summary, the ability of this Pseudomonas species to utilize crude petroleum aerobically, but not anaerobically, was reaffirmed. It was also obvious that when degradation of the petroleum occurred, extraction of the residual oil by floatation was impossible. Future extractions would have to be by use of n-pentane. The final point that was concluded from this experiment was that oil levels used here, and previously, were too high to readily detect utilization. In many cases, growth would have ceased long before the petroleum became a limiting substrate. Therefore subsequent utilization experiments were carried out with much lower oil concentrations, i.e. 0.1% of the incubation volume.

IV. Efficiency of n-Pentane Extraction as a Method of Residual Oil Recovery

As mentioned in Materials and Methods VII, for most of the work carried out in this investigation residual petroleum was extracted from cultures by the use of n-pentane. Because of this, a control experiment was carried out to ensure n-pentane extraction was capable of

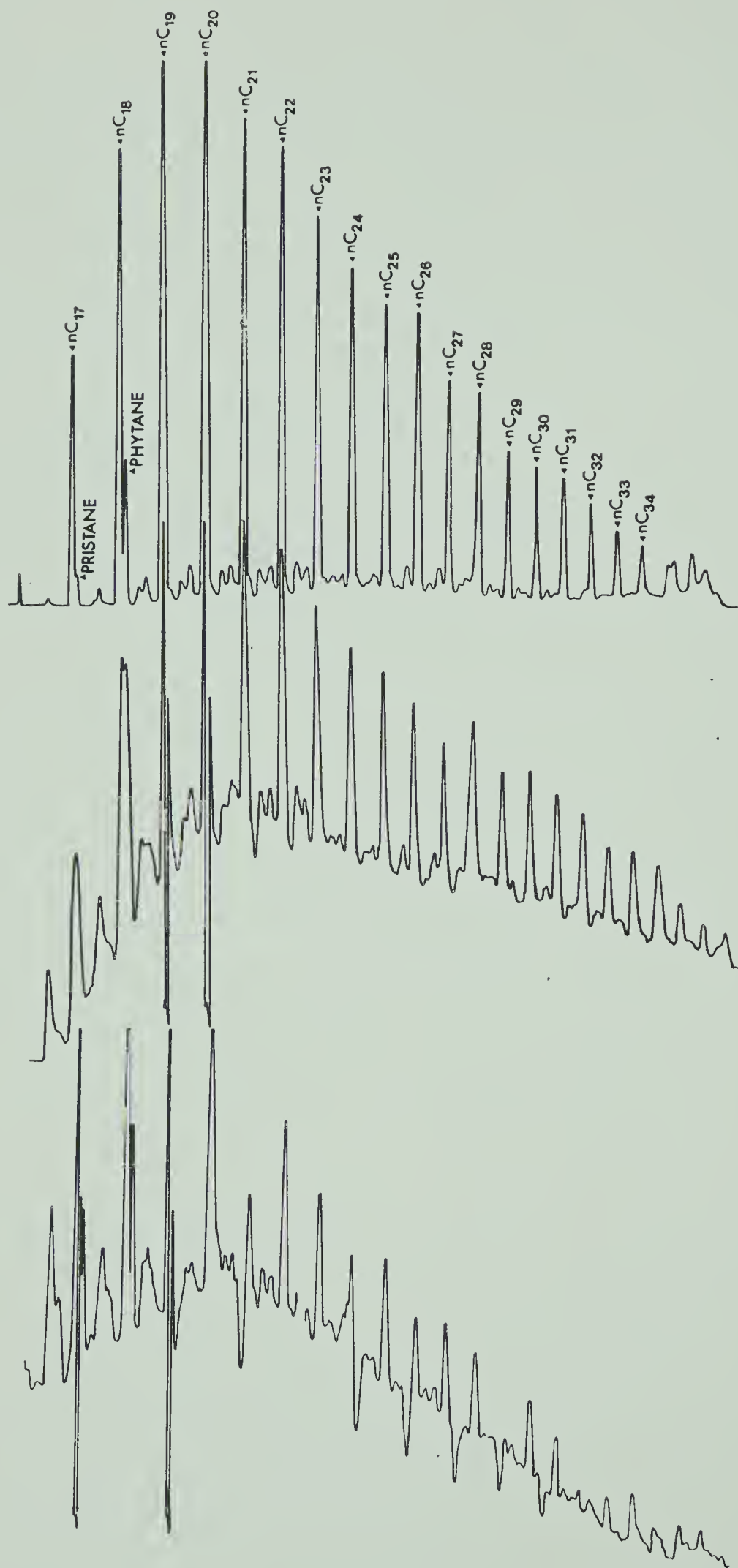


FIGURE 1

GAS CHROMATOGRAPHIC SATURATES ANALYSES OF STANDARD
AND RESIDUAL NORTH CANTAL OIL SAMPLES

Top: North Cantal standard saturates.

Middle: Residual saturates after aerobic metabolism
by the Pseudomonas sp.

Bottom: Residual saturates after anaerobic metabolism
by the Pseudomonas sp.

recovering all petroleum components from an aqueous environment, since, for the method to be successful, the recovered oil could not be altered due to losses by inefficient extraction.

For purposes of the test, two, 2 liter flasks containing 1.0 liter of sterile B+N salts/flask were each dosed with 1 ml of North Cantal oil. The flasks were shaken at 300 rpm (eccentricity, 1.0 inches) in a 30° incubator for 21 days. At the end of this shaking period, the flasks were extracted, one by pentane extraction, (Materials and Methods, VII) and the other by flotation and centrifugation. The residual oils as well as a sample of the original oil were subjected to liquid chromatography (Materials and Methods, VIII) and gas chromatographic analysis (Materials and Methods, IX). The liquid chromatographic results are presented in Table V.

It can be seen that reclaiming oil by floatation yields a fairly representative sample; but it must be remembered that the oil had not been subjected to bacterial growth. Thus the specific gravity of the oils had not exceeded 1.0. In the case of metabolized oils, however, floatation cannot be used as explained earlier in Results and Discussion and elsewhere in the text. Pentane extraction also allows full recovery of residual oil or oil from emulsions whether or not its specific gravity exceeds 1.0. At no time, either in terms of the values obtained from liquid chromatography of the residual oils or from GLC analyses of the residual

TABLE V

LIQUID CHROMATOGRAPHIC RESULTS OF CONTROL STUDY

Component	Oil by			Original control oil
	Flotation	n-C ₅	extraction	
	Weight %			
Benzene-soluble asphaltenes	3.6		2.5	3.5
Benzene-insoluble asphaltenes	10.7		6.8	6.9
Saturates	50.5		51.0	50.8
Aromatics	31.0		31.2	31.9
Soluble NSO	7.6		8.6	7.6
Insoluble NSO	0.0		0.3	0.5

All samples were from North Cantal stock oil.

All topping procedures carried out at 36.5°.

All values expressed as weight percentage of topped weight of oil.

saturate components, has n-pentane extraction showed evidence of severe bacterial component carry-over. For this reason, it was chosen as the superior method of oil recovery for subsequent experiments. A last conclusion arising from this experiment was that oils shaken in sterile media for long periods did not show any recognizable degradation after chromatographic analyses. Therefore, any degradation of oil detected in subsequent test systems must be attributed to bacterial action.

Since all three GLC profiles of the saturate fractions from the flotation sample, the n-pentane extraction sample and the original control sample, are identical and complete, it seems unnecessary to present them in this section.

V. Enrichment of a Mesophilic Soil Mixture with Hydrocarbon-Utilizing Abilities

As mentioned earlier in Materials and Methods III, a soil mixture with hydrocarbon-utilizing abilities at 30° was obtained by elective culturing technique using a sample of christmas tree soil as starting inoculum. It was thought that a mixture of bacteria might produce more marked changes in petroleum, since such a mixture would probably possess greater overall biochemical capabilities than would any single isolate.

The enrichment procedure was carried out as described in Materials and Methods III and V. The initial growth

pattern in the three original growth flasks is presented in Figure 2. For all subsequent experiments, North Cantal petroleum was provided at the rate of 1 ml/1,000 mls B+N salts medium.

Only a cursory identification of the components of the bacterial mixture was made; since it was felt that a study of the biochemical action of the mixture on petroleum was more important than a comprehensive description of the organisms. However, based on growth characteristics and microscopic examination, it would appear that the mixture was composed of a Pseudomonas species (different from 6c(b)100 described earlier in the text), a Flavobacterium species, an Achromobacter species and perhaps a Bacillus species either as an incidental contaminant or as an established member of the mesophilic mixture.

Photographs of the bacterial population are presented in Figure 3 before and after soil enrichment with North Cantal oil as a carbon substrate. This is merely to illustrate the stimulation of a zymogenous population having direct fermentative abilities on crude petroleum as compared with the original autochthonous population. Where enrichments were carried out using 5 and 10 mls of oil/liter of B+N salts medium, death of the original population due to oil toxicity is especially evident, and is reflected in the viable counts portrayed in Figure 2. This decrease in viable count reversed itself only after the zymogenous

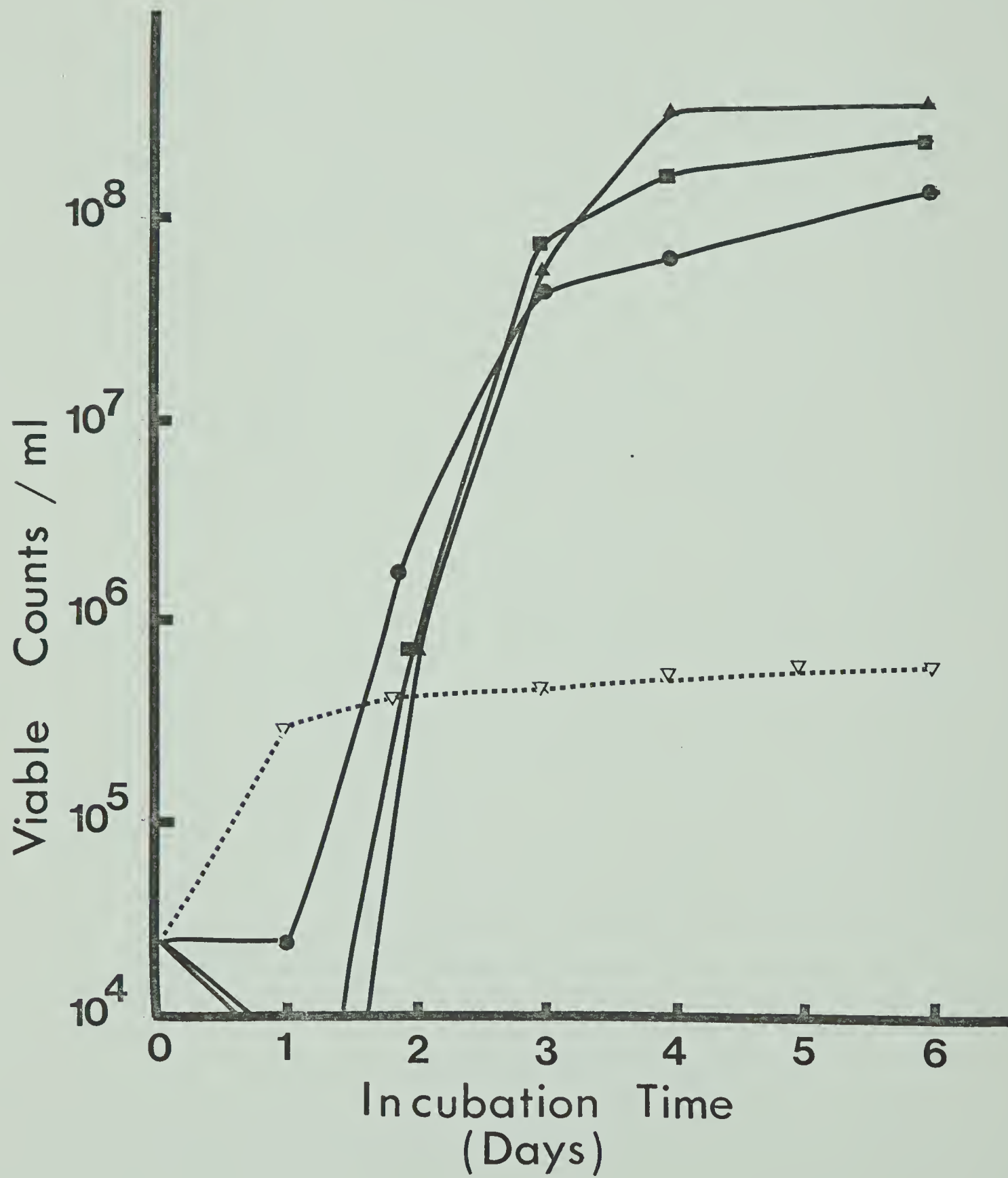


FIGURE 2

GROWTH OF A 10% CHRISTMAS TREE SOIL SUSPENSION
 INOCULATED INTO B+N SALTS + NORTH CANTAL OIL

The method by which this enrichment or elective technique was set up is described in the text.

Graph: Viable counts/ml vs. Incubation time (days)

B+N salts + 1.0 ml North Cantal oil/l.	(—○—○—)
B+N salts + 5.0 ml North Cantal oil/l.	(—■—■—)
B+N salts + 10.0 ml North Cantal oil/l.	(—▲—▲—)
B+N salts control	(...▽...▽...)

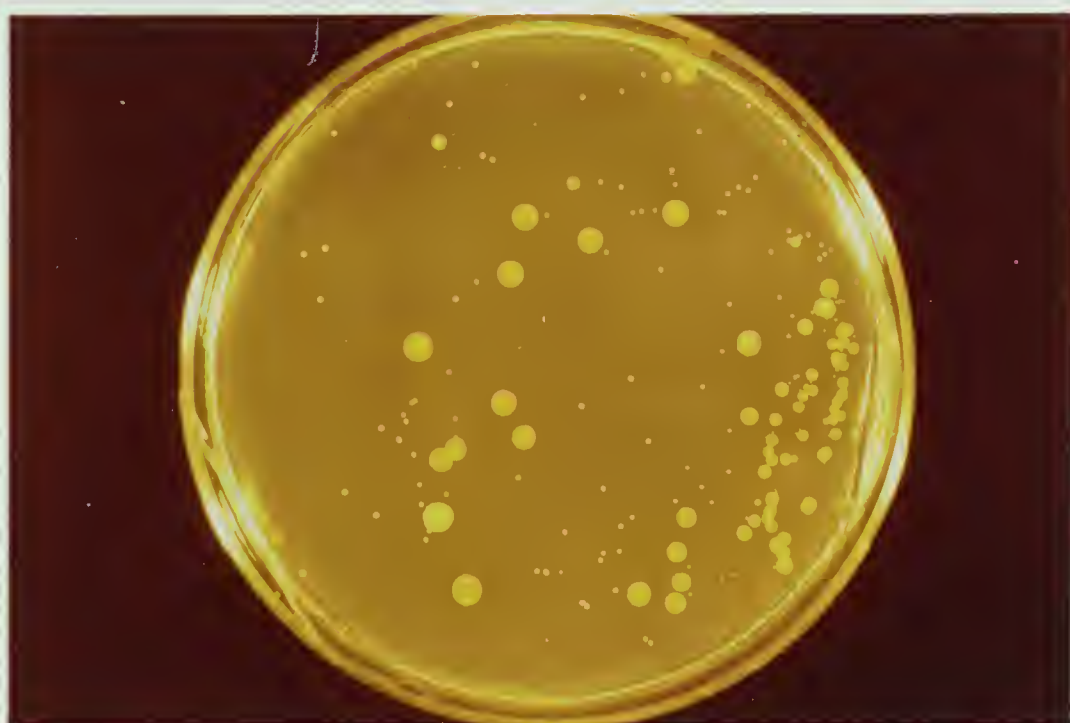
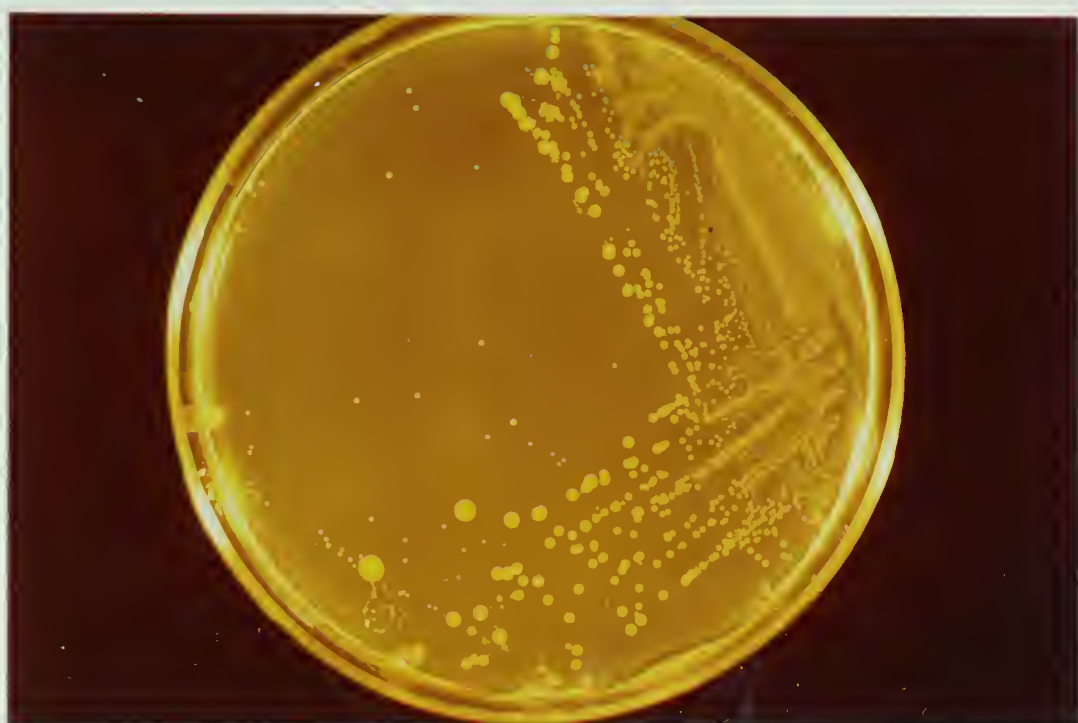


FIGURE 3

PHOTOGRAPHS SHOWING BACTERIAL
ADAPTATION TO GROWTH ON CRUDE PETROLEUM

Top: Photograph showing zymogenous bacterial population obtained by enrichment on B+N salts + 1.0 ml North Cantal oil/liter of culture.

Bottom: Photograph showing the original autochthonous soil population.

population reached the log phase of growth.

It is rather evident that if a soil inoculum for enrichment culture has had any previous exposure to petroleum, the period of time needed for selection of organisms which grow well on oil components does not extend much beyond 24-48 hours. It was found that the stabilized soil mixture, maintained on B+N salts and petroleum, showed a lag phase of less than 24 hours when inoculated into fresh B+N salts and petroleum. This lag period was extended if the inoculum was more than a few days old. Figure 2 does show a relationship between population response and amount of oil added, especially at day 4.

VI. Sequential Studies Concerning the Utilization of North Cantal Oil by the Mesophilic Soil Mixture

After stabilization of the mesophilic soil mixture from the christmas tree site soil, it was necessary to gain a more comprehensive insight into the mixture's capabilities in terms of petroleum degradation. Most important, was the necessity to determine whether metabolism of the North Cantal oil by the mixture would yield a residual petroleum with characteristics more closely related to the degraded oils found at the opposite end of the Weyburn field.

A 21 day sequential utilization experiment was designed, using B+N salts medium + 0.1% North Cantal oil, and the mesophilic christmas tree soil mixture as inoculum. The procedures for carrying out the experiment were as described

in Materials and Methods V. At specified times, throughout the three week incubation period, single flasks were removed from the experiment, at random, and n-pentane extracted as described in Materials and Methods VII. In addition, as flasks were taken to be harvested, the pH was also determined, before extraction, to determine if trends of acidity or basicity could be established.

The viable counts, also obtained during the incubation period are presented in Figure 4. The aqueous pH trends established during the incubation period are shown in Figure 5. Results of liquid chromatographic analyses appear in Table VI.

The viable count reaches a maximum at $1.1-1.4 \times 10^8$ viable cells/ml. This appears to be a constant quality of the soil mixture growing under the above conditions. The maximum viable count (stationary phase) remains constant for at least 7 days before the death rate comes noticeable. Therefore, either the cells are remaining viable for long periods of time, but not undergoing growth, or the growth rate is matching the death rate for this period of time. Since further degradation of the saturate fraction is occurring during this time, the latter reason would appear to be the more logical one, unless the cells in the stationary phase are maintaining a high level of endogenous metabolism.

The net pH trend during active growth appears to be an

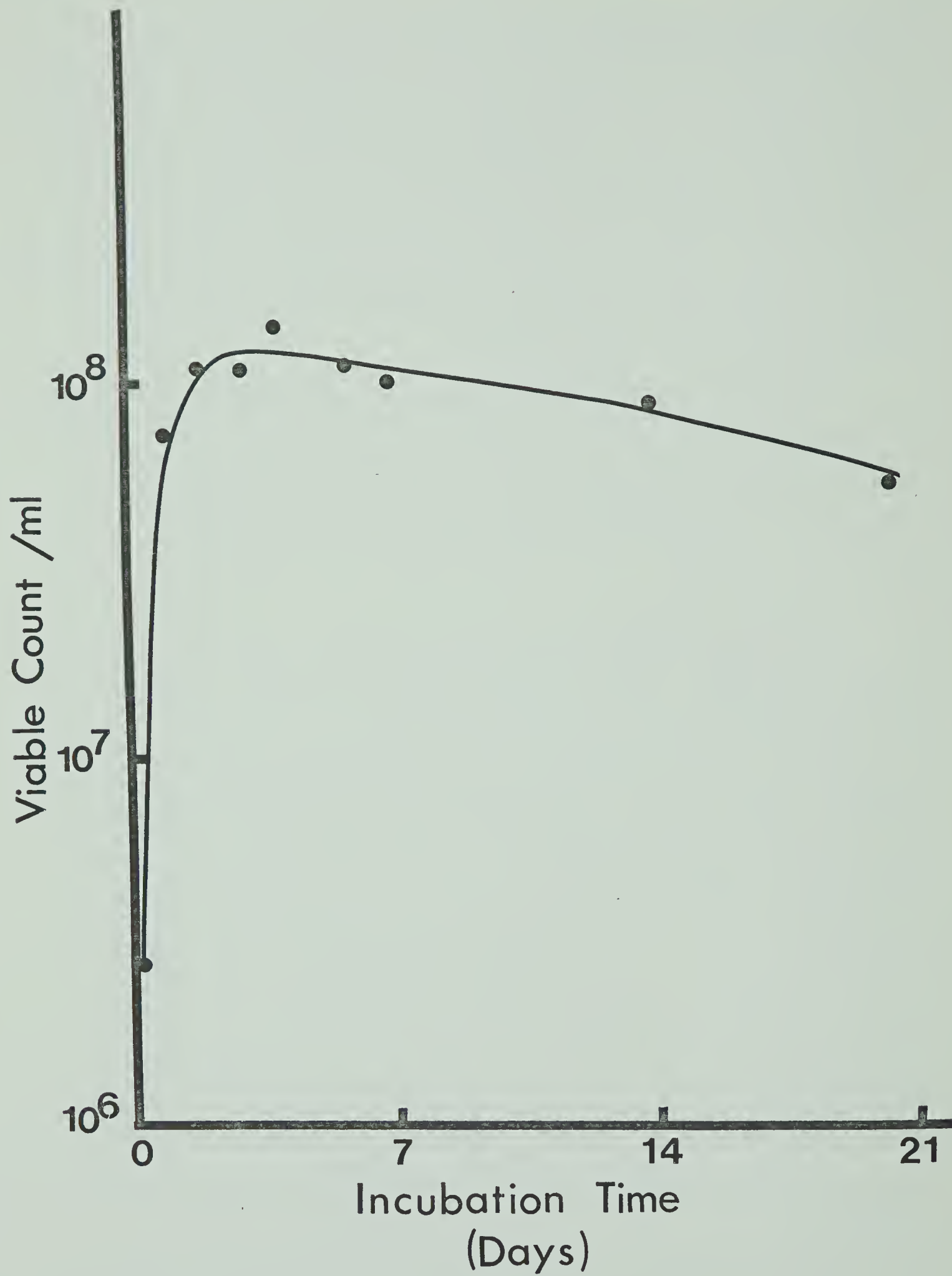


FIGURE 4

VIABLE COUNTS OF SOIL MIXTURE ORGANISMS INCUBATED
WITH B+N SALTS + 0.1% NORTH CANTAL OIL

Procedures by which this experiment was carried out are presented in the text. The method of enumeration by plate count is described in Materials and Methods VI.

Graph: Viable count/ml of incubation mixture vs.
Time (days).

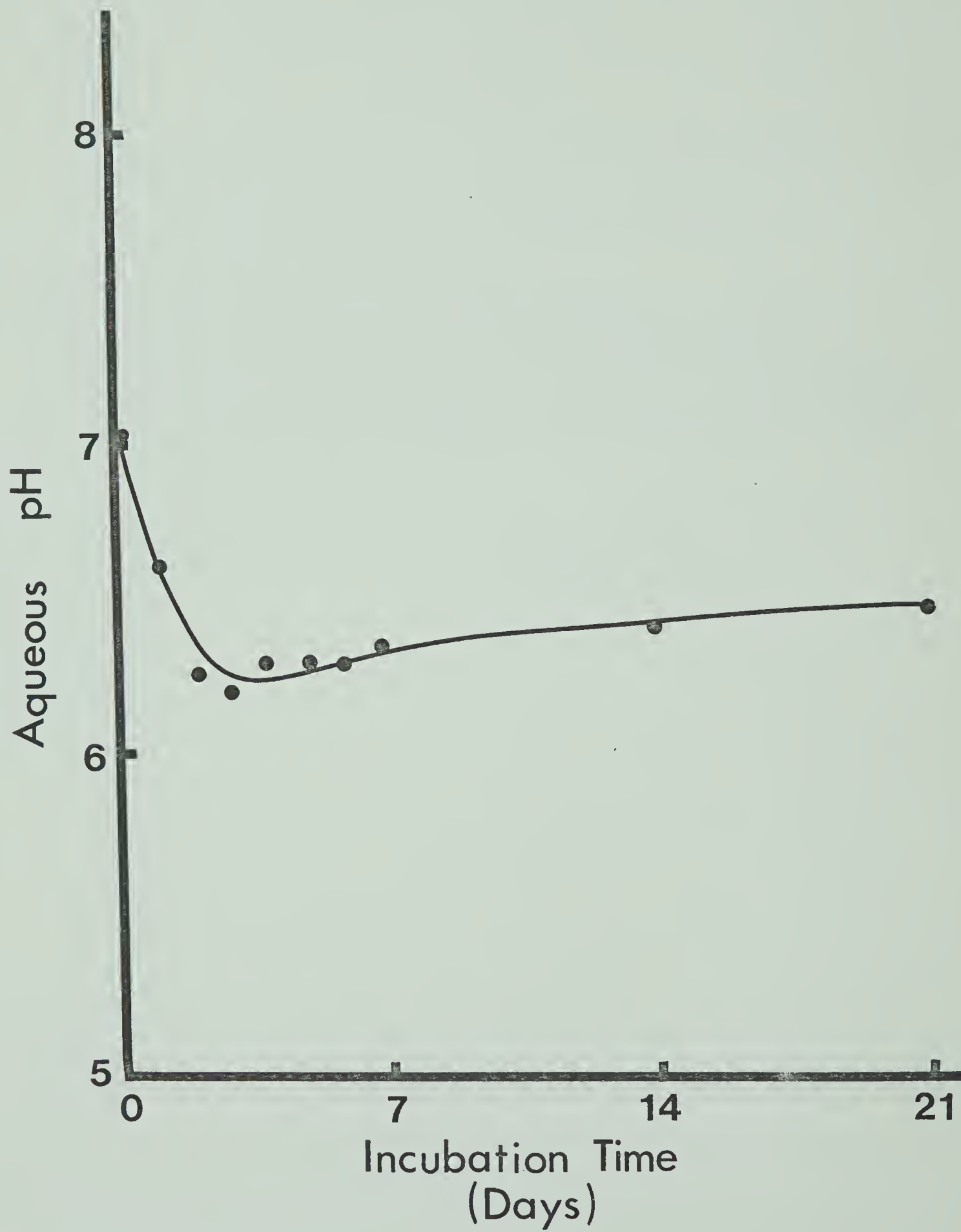


FIGURE 5

THE INFLUENCE ON pH OF SOIL MIXTURE ORGANISMS
INCUBATED WITH B+N SALTS + 0.1% NORTH CANTAL OIL

One ml aliquots of the complete incubation mixtures were taken at the times indicated on the graph, and the aqueous pH was determined.

Graph: Aqueous pH vs. Time (days).

LIQUID CHROMATOGRAPHIC ANALYSES OF RESIDUAL
PETROLEUM FROM SOIL MIXTURES

All values expressed as weight percent of topped weight.
Topping temperature: 32°.

acidic one with slight reversal toward the end of the incubation period. This could be taken to mean that free fatty acids are to be found within the aqueous phase during active growth, or that acidic end-products are actually being produced by the soil mixture. The reversal of the acidic trend after day 14 is probably due to cell autolysis which usually yields an increase in alkalinity in most bacterial systems.

The liquid chromatographic analyses does, for the most part, illustrate certain consistent changes in the petroleum composition as a result of bacterial alteration. The definite decrease in saturates content is probably a reflection of the fact that apart from certain non-hydrocarbon components possibly being present, the saturate fraction is biochemically the most straightforward group of compounds to oxidize and utilize. This is due to the fact, that once an n-alkane is oxidized, β -oxidation yields two-carbon acetate moieties which can easily enter the central metabolic routes of the organism to produce energy. All other components within crude oil require multiple, endergonic, reactions to split and cleave aromatic and cyclic ring structures, before any energy-yielding processes can occur. In looking at the GLC profiles of the sequential saturate samples (Figure 6), two things become apparent. The first point is that pristane and phytane are less amenable to biological attack, and only disappear after the

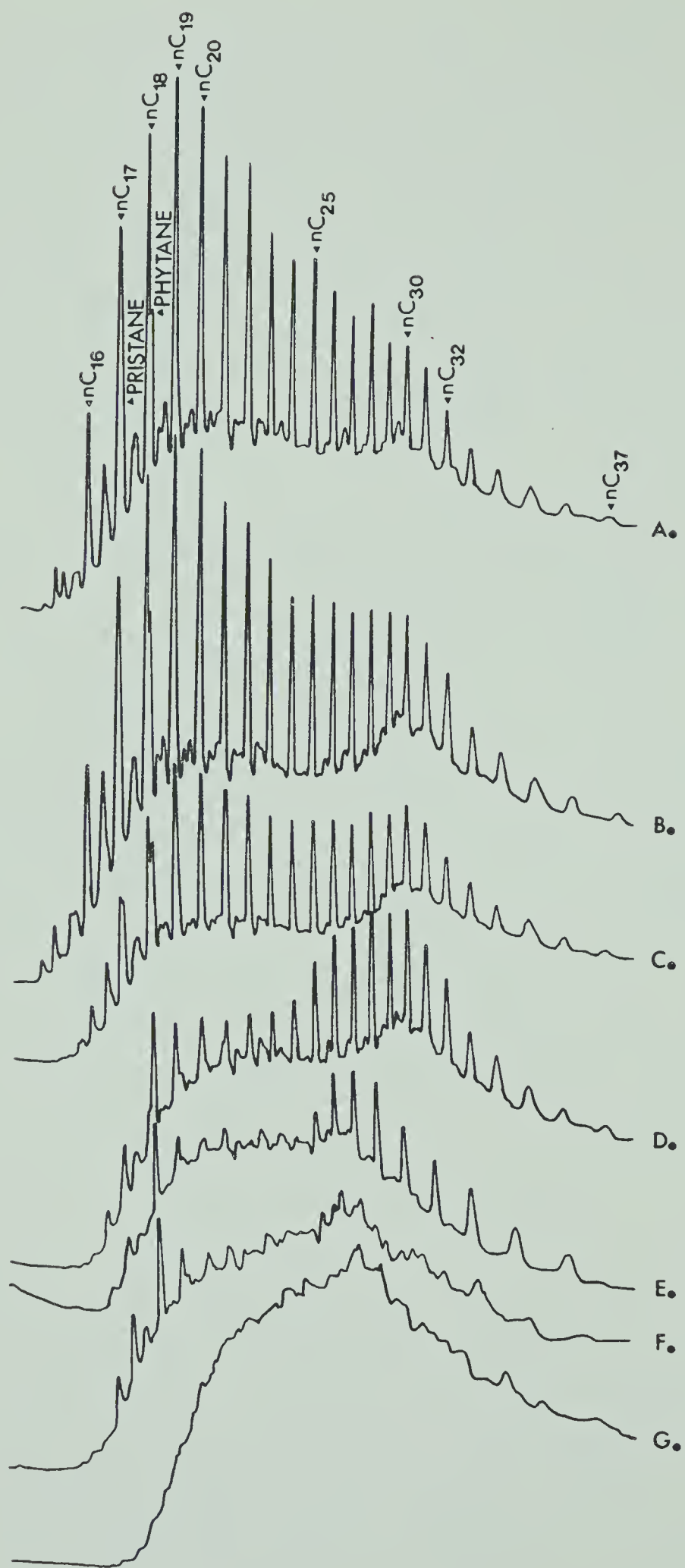


FIGURE 6

GLC ANALYSES OF RESIDUAL SATURATE FRACTIONS
FROM SEQUENTIAL UTILIZATION BY THE
CHRISTMAS TREE SOIL MIXTURE

- A. Control saturates of North Cantal oil.
- B. Day 2 saturates.
- C. Day 3 saturates.
- D. Day 4 saturates.
- E. Day 5 saturates.
- F. Day 14 saturates.
- G. Day 21 saturates.

comparatively less-refractive n-saturates have been utilized. Nevertheless they are eventually utilized, leaving only an iso-and cycloparaffin envelope remaining after 21 days of incubation.

The consistent observation made throughout the course of the work with the mesophilic soil mixture is that at some point in their growth, the organisms have made use of all n-saturates from nC_{16} to nC_{24} . However, from nC_{25} to the highest of the n-saturates, there seems to be a lag in their utilization. No suitable explanation of this phenomenon can be made at this point. Other workers have shown that n-decane can undergo cyclization, thus becoming resistant to utilization (Van Der Linden and Thijsse, 1965b), while n-nonane and undecane always have at least one free end open to attack. Such a mechanism cannot explain the above observation, since the whole series of saturates from nC_{25} onwards are affected; and it seems unlikely that all the series would have their terminal ends in a buried or blocked configuration. Perhaps the late utilization of nC_{25} and higher saturates indicates a specificity of transport or an upper limit to the specificity of the active sites on the initially-induced oxidative enzymes.

A final problem arises in the interpretation of the results of this sequential utilization. By assuming only the saturate components of the petroleum are being utilized, one negates the very possibility that enrichment or

utilization of the other components could in fact have occurred. It was therefore decided to attempt to calculate the theoretical enrichment of the other petroleum components, had the saturates been the only fraction utilized. The values thus obtained were compared with the values actually obtained after extraction and chromatography of the day 21 sample. The theoretical and actual results are presented in Table VII. Although variance appears to be serious within certain samples, a few facts cannot be ignored. It appears the soil mixture had no degradative capabilities against the benzene-insoluble asphaltenes or the soluble NSO components. It would also seem that some form of metabolic end-products were in fact added to the soluble asphaltenes as well as the insoluble NSO fraction. Only the aromatic fraction shows reasonable evidence of being utilized to some extent.

VII. The Influence of Aeration on Utilization of North Cantal Oil by the Christmas Tree Soil Mixture

A great deal of the literature concerning hydrocarbon utilization stresses the importance of aeration as a factor in maximizing cell yield when using such substrates (Casida, 1968).

In the course of this investigation, an experiment was carried out, using the mixed culture, to determine the influence of aeration on growth as well as hydrocarbon utilization. The design of the experiment, as presented in

TABLE VII

THEORETICAL AND ACTUAL RESIDUAL CHROMATOGRAPHIC ANALYSIS OF NORTH CANTAL OIL
AFTER SOIL MIXTURE METABOLISM

Component	Original	Theoretical	Actual
	analysis	residual analysis	residual analysis
		Weight %	
Benzene-soluble asphaltenes	2.5	3.3	5.1
Insoluble asphaltenes	6.8	8.9	8.1
Saturates	51.0	35.6	35.6
Aromatics	31.2	41.9	36.8
Soluble NSO	8.6	11.2	10.9
Insoluble NSO	0.3	0.4	3.3

All values expressed as weight-percentages of the original topped weight.

Materials and Methods V, attempted to leave aeration as the only variable in the system. Four, two liter flasks, containing 250, 500, 750, and 1,000 mls of B+N salts, were inoculated to the same initial cell concentration and were provided with North Cantal oil to a concentration of 0.1%. Viable counts, and pH determinations were carried out initially, and at subsequent daily periods for 5 days.

As an additional check of how much oxygen was in fact being dissolved in the various incubation volumes, a sulfite oxidation test was undertaken according to the procedure given in Standard Methods FOR THE EXAMINATION OF Water and Wastewater, pp. 244-245. All resulting data from the sulfite oxidation test was converted to mgs SO_3^- oxidized in 60 minutes, and is presented in Figure 7.

From 250 to 750 mls of B+N medium in a two liter flask seems to cause a linear decrease in dissolved O_2 under agitated conditions. This effect levels off, however, between 750 and 1,000 mls. Thus bacteria metabolizing in this range of volumes, are under similarly low levels of aeration.

At the end of the incubation period for the actual growth flasks, all were extracted with equivalent amounts of n-pentane as per Materials and Methods VII. These residual petroleums were then subjected to liquid and gas chromatographic analyses.

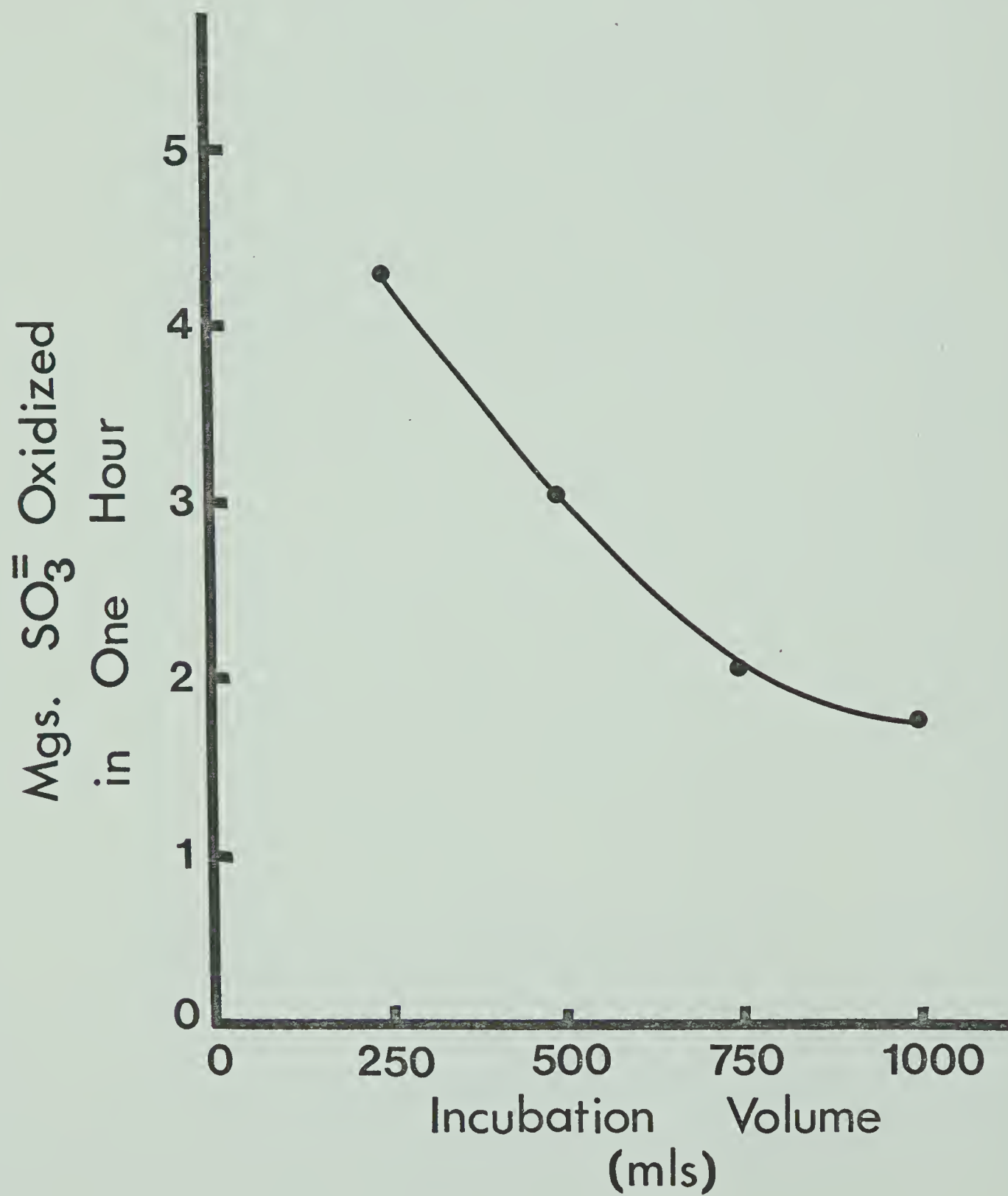


FIGURE 7

INFLUENCE OF VOLUME OF LIQUID USED PER FLASK
ON AMOUNT OF DISSOLVED O₂ IN SOLUTION

The method is as described in the text of the thesis. This experiment applies only to flasks containing B+N salts and no petroleum or cells. The possible influence of the latter two factors, if added, is not known.

Results of the viable counts presented in Figure 8 indicate that aeration is not a prime consideration when one is discussing the growth rate of this particular soil mixture on petroleum. There is some initial evidence that an abundance of O_2 , as in the case of the 250 ml flask, retards the log phase of growth. However, all flasks produced growth in excess of 10^8 /ml, so this effect of initial retardation is not a permanent problem.

The results of liquid and gas chromatographic analysis, do show the influence of aeration (Table VIII and Figure 9). Within 5 days, growth under well aerated conditions in the 250 ml flask, had reduced the saturate percentage to roughly 36% from the original 51%. From 500 to 1,000 mls/flask, the effect of aeration, or lack of it, did not produce significant differences. However, it was noticed that even in the 1,000 ml flask, the amount of saturates utilized within 5 days was actually less than expected when one compares this experiment with others quoted earlier in the text using the same soil mixture. This could have been due to age problems within the original inoculum used. Nevertheless, reduction of the saturates to less than 36% of the topped weight in five days had never been accomplished with the same soil mixture before, using larger volumes for incubation. Therefore the difference exhibited in saturates utilization between the 250 ml flask and the others is real and significant.

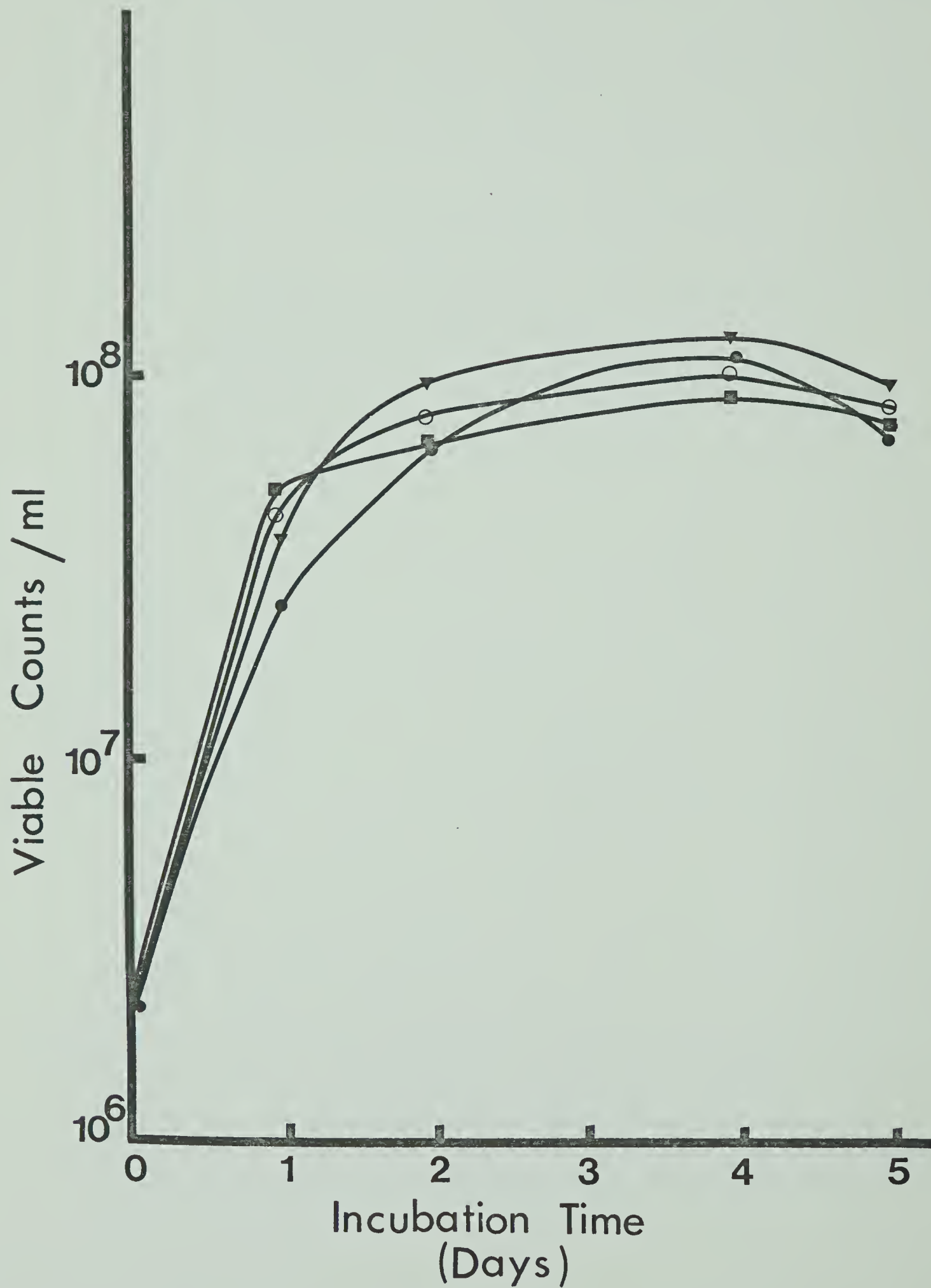


FIGURE 8


THE INFLUENCE OF AERATION LEVELS ON THE GROWTH
 RESPONSE OF THE CHRISTMAS TREE SOIL MIXTURE
 IN B+N SALTS + 0.1% NORTH CANTAL OIL

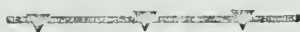
This particular growth experiment was carried out as described in the text. The method of plating was as presented in Materials + Methods VI.

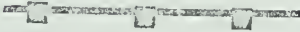
Initial viable count: $2.8 \times 10^6/\text{ml}$.

Graph: Viable Counts/ml of Incubation

Mixture vs. Incubation Time (Days)

250 mls: ()

500 mls: ()

750 mls: ()


1000 mls: ()

TABLE VIII
RESULTS OF LIQUID CHROMATOGRAPHIC ANALYSIS OF
RESIDUAL OILS FROM AERATION EXPERIMENT

Component	Volume in Incubation Flasks (mls)				Control
	250	500	750	1000	
	Weight %				
Benzene-soluble asphaltenes	7.6	4.8	3.3	3.3	2.5
Benzene-insoluble asphaltenes	6.1	6.8	5.0	5.5	6.8
Saturates	35.9	46.8	45.6	47.2	51.0
Aromatics	34.8	32.5	32.3	32.9	31.2
Soluble NSO	14.4	10.7	10.2	10.1	8.6
Insoluble NSO	1.0	1.0	3.2	1.0	0.3

Topping temperature: 32°.

All values are weight percentages of the topped weight.

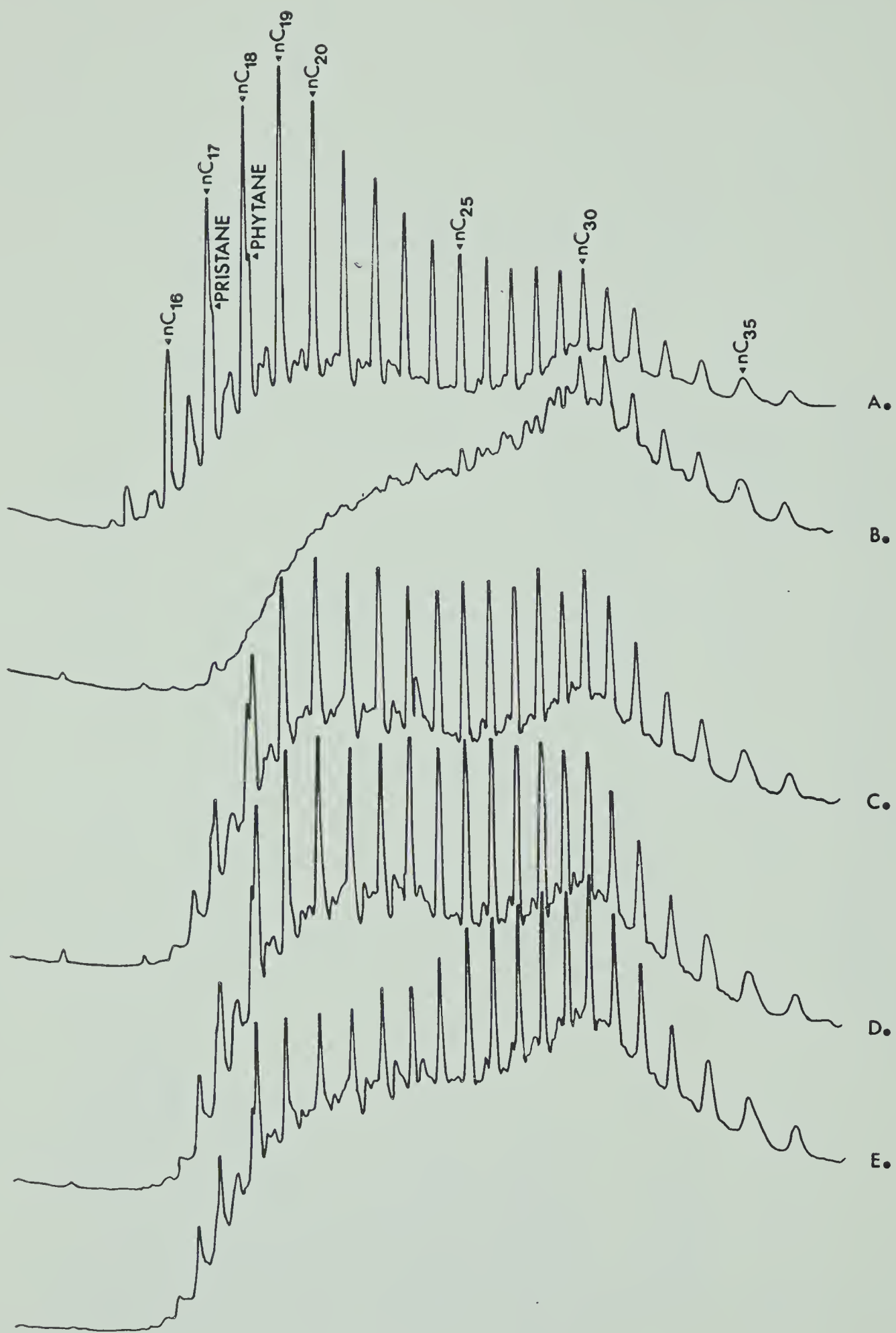


FIGURE 9

RESIDUAL SATURATES PROFILES OF SAMPLES
RECOVERED FROM AERATION EXPERIMENT

- A. North Cantal control saturates.
- B. Residual saturates from 250 ml flask.
- C. Residual saturates from 500 ml flask.
- D. Residual saturates from 750 ml flask.
- E. Residual saturates from 1000 ml flask.

The pH trends, (Figure 10) also indicate greater utilization of hydrocarbons within the 250 ml flask. This conclusion is due to the fact that typically, good hydrocarbon utilization is synonymous with a lowering of the pH as mentioned earlier in the text. In total, therefore, this experiment seemed to show a lack of influence of aeration on increase in viable counts, but a significant influence on the degree of petroleum degradation. In reviewing Table VIII, it is evident that utilization of saturates in the 1,000 ml incubation volume had just begun at the fifth day and slight aromatic enrichment had occurred. In the case of the 250 ml incubation volume, much saturate utilization had taken place. If only the saturates had been used in this case, the aromatic fraction should have been enriched to over 40% of the topped weight of residual oil. Yet, the aromatic fraction only increased by roughly 2% of the residual topped weight. It would seem that under the aeration conditions of the 250 ml incubation volume, secondary aromatic utilization can be significant; while in the one liter incubation volume it is not.

The apparent utilization enhancement created by using 250 ml incubation volumes would make it appear that use of 1 liter incubation volumes in previous and subsequent experiments was not sound technique. It must be remembered, however, that the purpose of this investigation was to

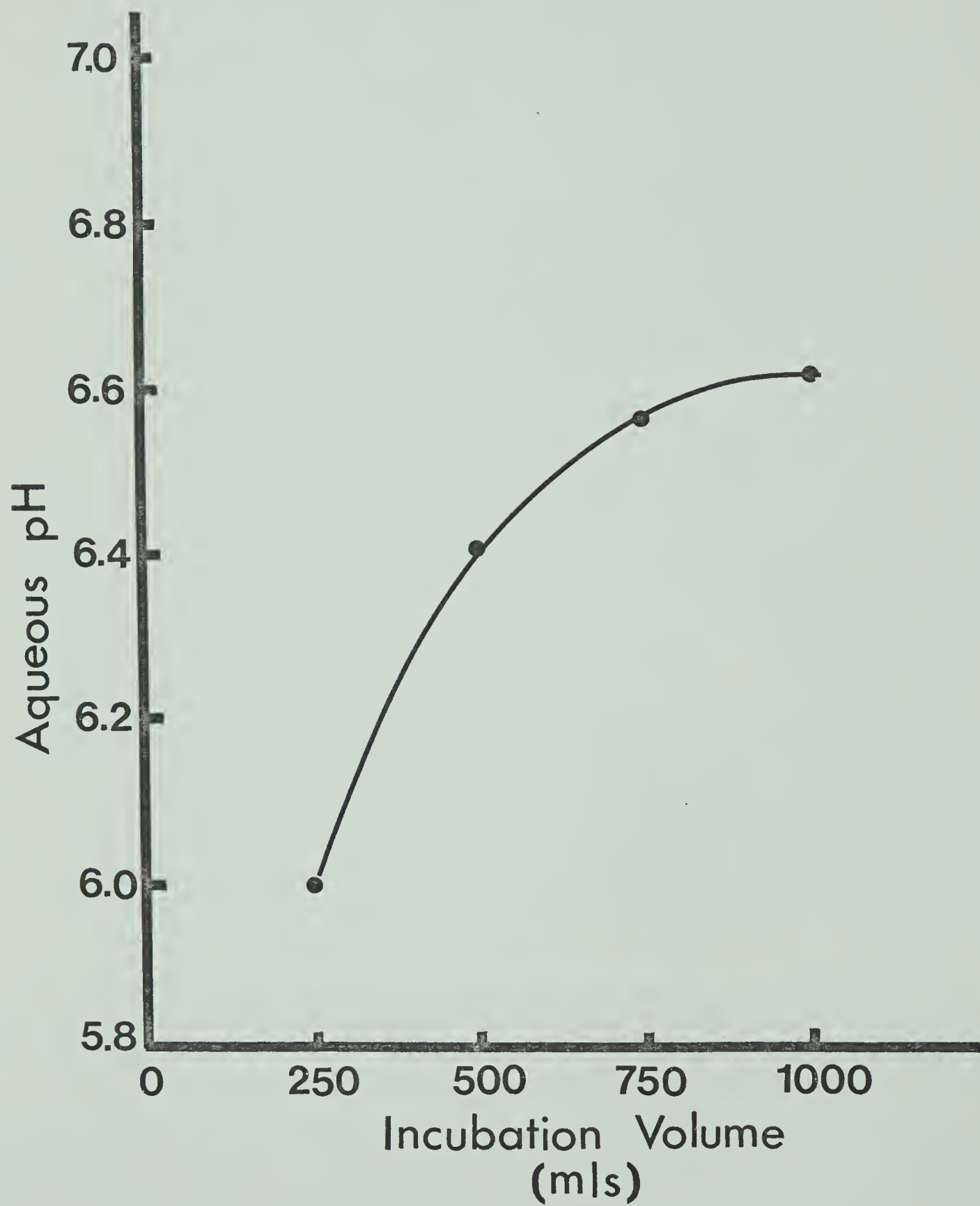


FIGURE 10

INFLUENCE OF INCUBATION VOLUME ON pH VALUES
RESULTING FROM PETROLEUM METABOLISM
BY THE CHRISTMAS TREE SOIL MIXTURE

At the end of the 5 day incubation period, as described in the text, and before n-pentane extraction, samples were removed from the flasks and their pH was determined. Initial pH of the incubation flasks containing B+N salts, petroleum, and fresh inoculum was 7.05.

metabolize the superior North Cantal oil with bacterial isolates and mixtures, in an attempt to create an oil with chromatographic properties approaching Stoughton or Lost Horse Hill oils. The latter two oils are relatively high in aromatic character and low in saturate character. Therefore it seems more logical to do experimental work in one liter volumes (or equivalent volumes); and incubate for longer periods of time to finally obtain oils with lower saturate character and higher aromatic character (see Table VI). Even these conditions did allow some aromatic degradation as shown previously in Table VII. However, the aeration conditions of a one liter volume are more realistic than those of a 250 ml volume when one is extrapolating to a subterranean environment as is the case in an oil deposit. The enhancement of the degradative process under more optimum aeration conditions is only a logical extension of the known facts.

VIII. Recycling of Residual North Cantal Oil by the Mesophilic Christmas Tree Soil Mixture

In previous experiments, it has been shown that North Cantal oil, when provided at a volume:volume concentration of 0.1% in B+N salts allowed the soil mixture to grow to a cell concentration of $1-2 \times 10^8$ /ml (Figure 4). At the same time the saturate component of the petroleum was lowered to 35 - 36% of the topped residual weight

(Table VI). Gas chromatographic analysis at this point usually showed a complete absence of n-saturate peaks (Figure 6). Any attempts to induce the same population to grow on the residual petroleum once again by addition of fresh B+N salts, etc., were without success. Therefore it was assumed that once the n-saturates were utilized, the petroleum could not be used as growth substrate for the same soil population. However, if the soil mixture was provided with North Cantal oil in concentrations of 0.5% (5 mls/liter of B+N salts) and allowed to grow to a maximum viable count; the residual petroleum still showed n-saturate peaks in its GLC profile. It was felt that this residual oil should be capable of supporting further growth by the christmas tree soil mixture. To test this hypothesis, the following experiment was carried out. In order to generate residual oil for the critical portion of the experiment, two, one liter volumes of B+N salts + 0.5% North Cantal oil were inoculated with the christmas tree soil mixture, and incubated for seven days according to the general scheme laid out in Materials and Methods V. Figures 11 and 12 show the results of viable count and pH measurements carried out during this initial part of the experiment. It is important to note that, in both flasks the residual petroleum readily floated on the surface of the salts medium, indicating a specific gravity still less than one. This can be taken to mean that very little

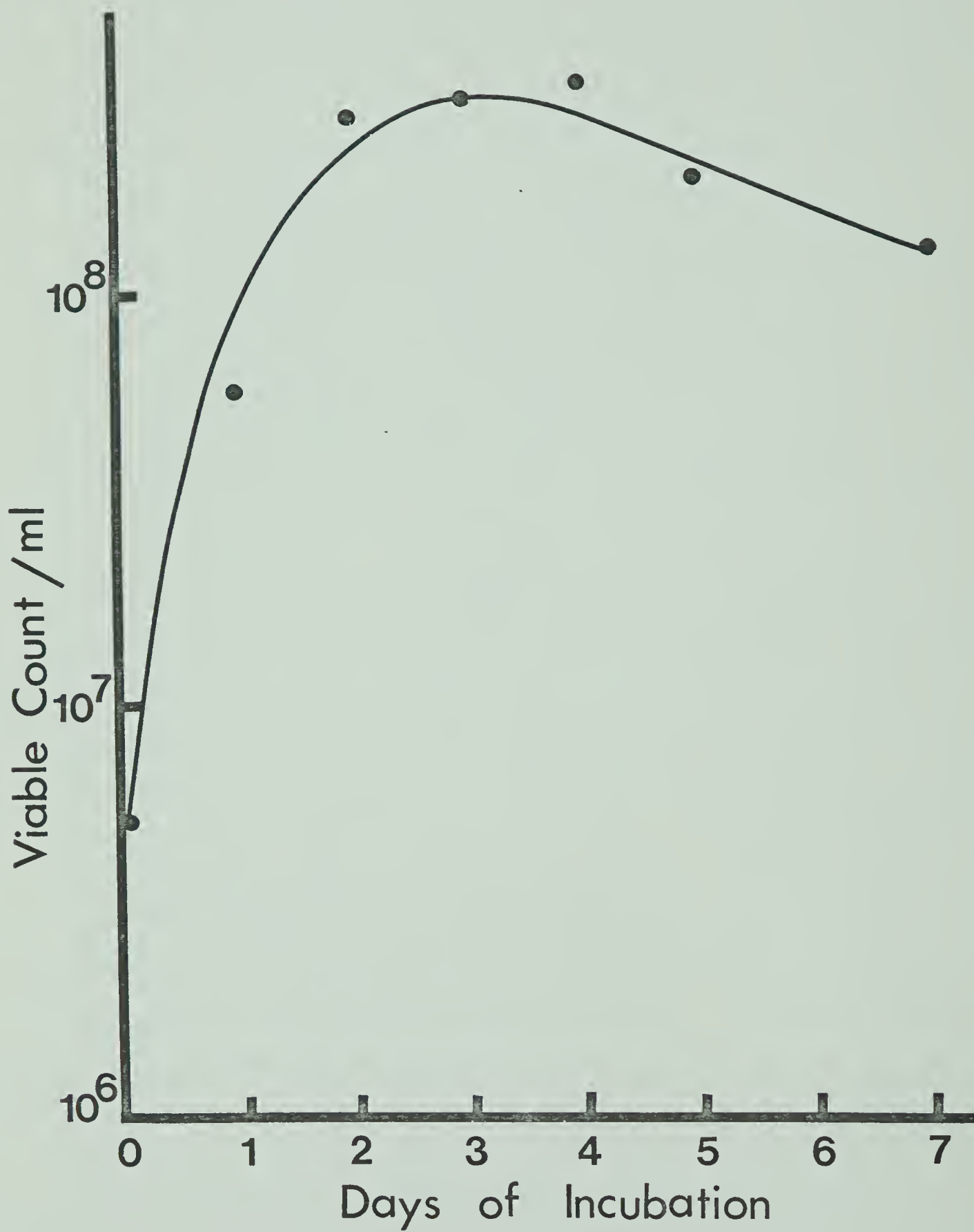


FIGURE 11

THE GROWTH RESPONSE OF CHRISTMAS TREE SOIL MIXTURE
IN B+N SALTS + 0.5% NORTH CANTAL OIL

Experimental methods were as described in the text.

Graph: Viable Counts/ml vs. Days of Incubation.

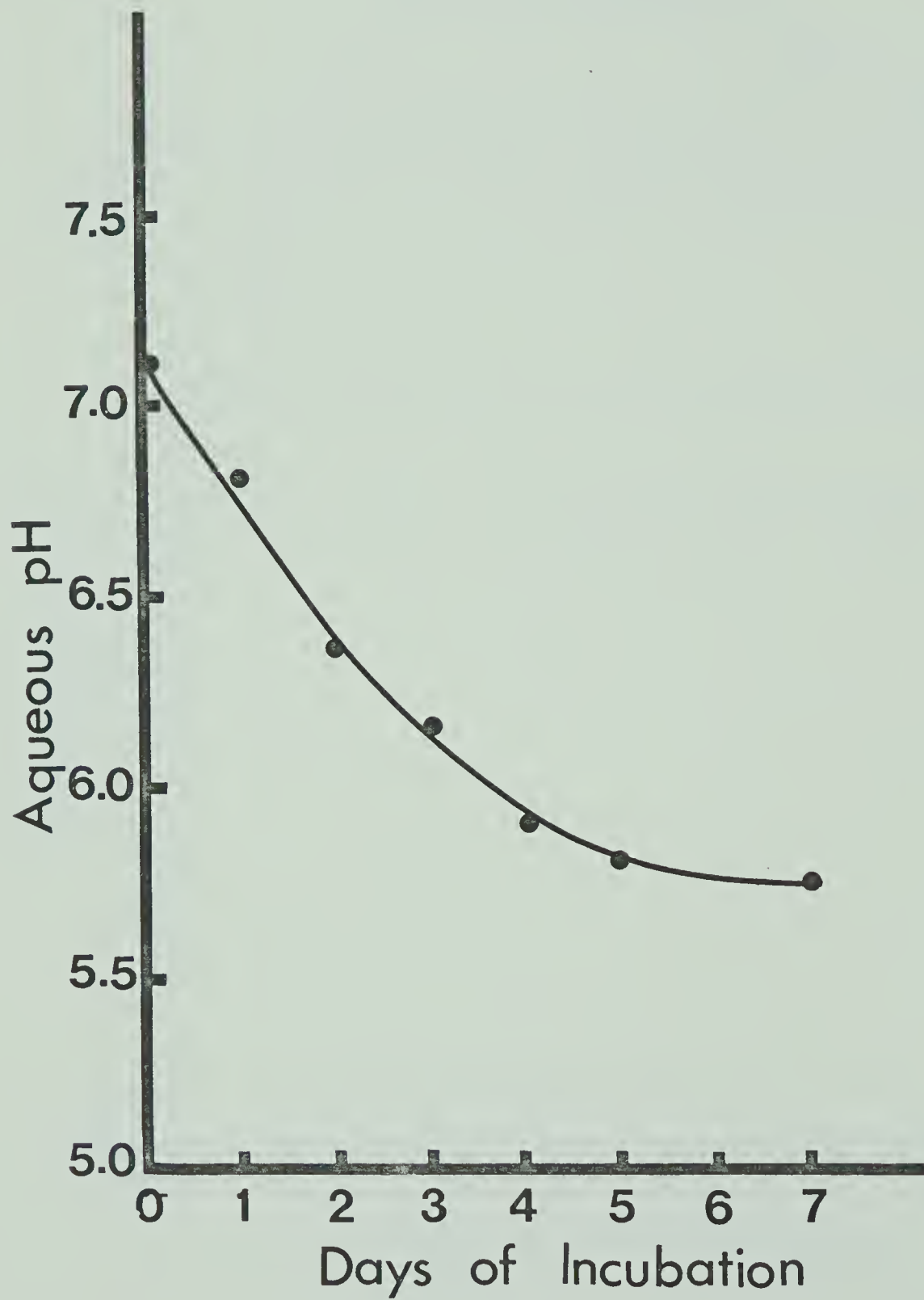


FIGURE 12

pH CHANGES OF CHRISTMAS TREE SOIL MIXTURE
GROWTH IN B+N SALTS + 0.5% NORTH CANTAL OIL

Aqueous pH values were determined daily
on 1.0 ml samples taken from one of the growth
flasks as described in the text.

Graph: Aqueous pH vs. Days of Incubation.

degradation had occurred to this point. The volumes were then combined, shook for a further one hour and then re-split into one liter quantities to ensure identical samples going into cycle 2 of the experiment. With the residual oils remaining at the surface of the liquids, 950 mls of aqueous material was removed from each flask and replaced with fresh B+N salts medium, to regenerate the same volume. One flask, now labelled Flask A, was prepared for incubation at this point, while the second flask, labelled Flask B, was given an additional 25 ml volume of a 10% soil suspension of the same christmas tree soil from which the original soil mixture had been isolated. Both flasks were then incubated for 21 days as described in Materials and Methods V. Figures 13 and 14 show plate count and pH measurements, made during this second cycle of growth.

At the end of the 21 day incubation period, the flasks were allowed to stand for 5 hours to detect any obvious density changes which might have occurred within the residual oil samples. Figure 15 shows the obvious changes which occurred in Flask B as opposed to Flask A. In Flask A, the specific gravity was still less than one (i.e. the petroleum floated) indicating no further significant attack of the residual petroleum had occurred in cycle 2. The obvious degradation which had occurred in Flask B in cycle 2, coupled with the significant pH drop is good

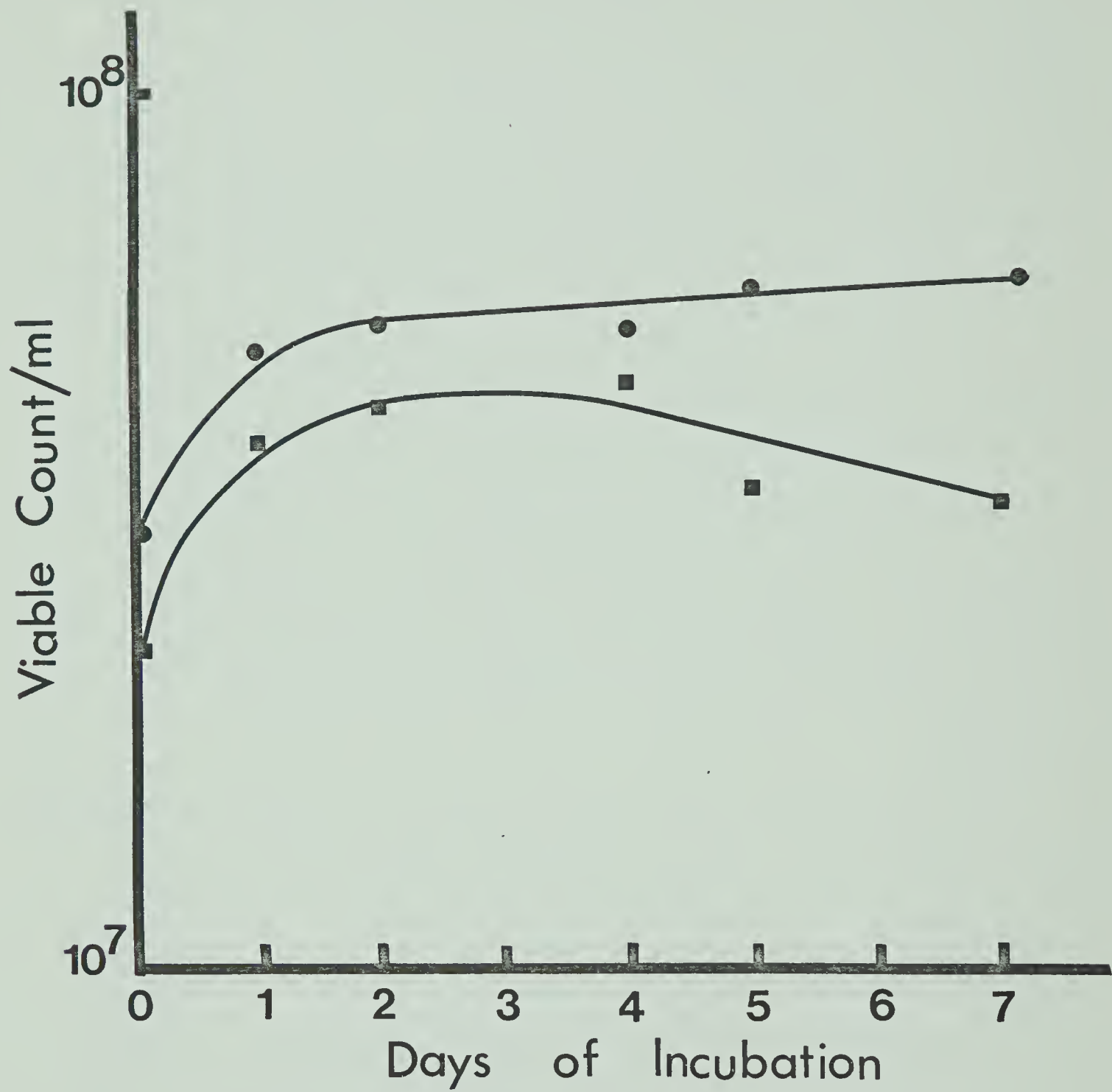


FIGURE 13

VIABLE COUNTS RESULTING FROM GROWTH ON RECYCLED PETROLEUM

Procedures were carried out as described in the text.

Graph: Viable counts/ml vs. Days of Incubation.

Flask A: Recycling of residual
petroleum with original
soil mixture

()

Flask B: Recycling of residual
petroleum with original
soil mixture + 10%
soil suspension

()

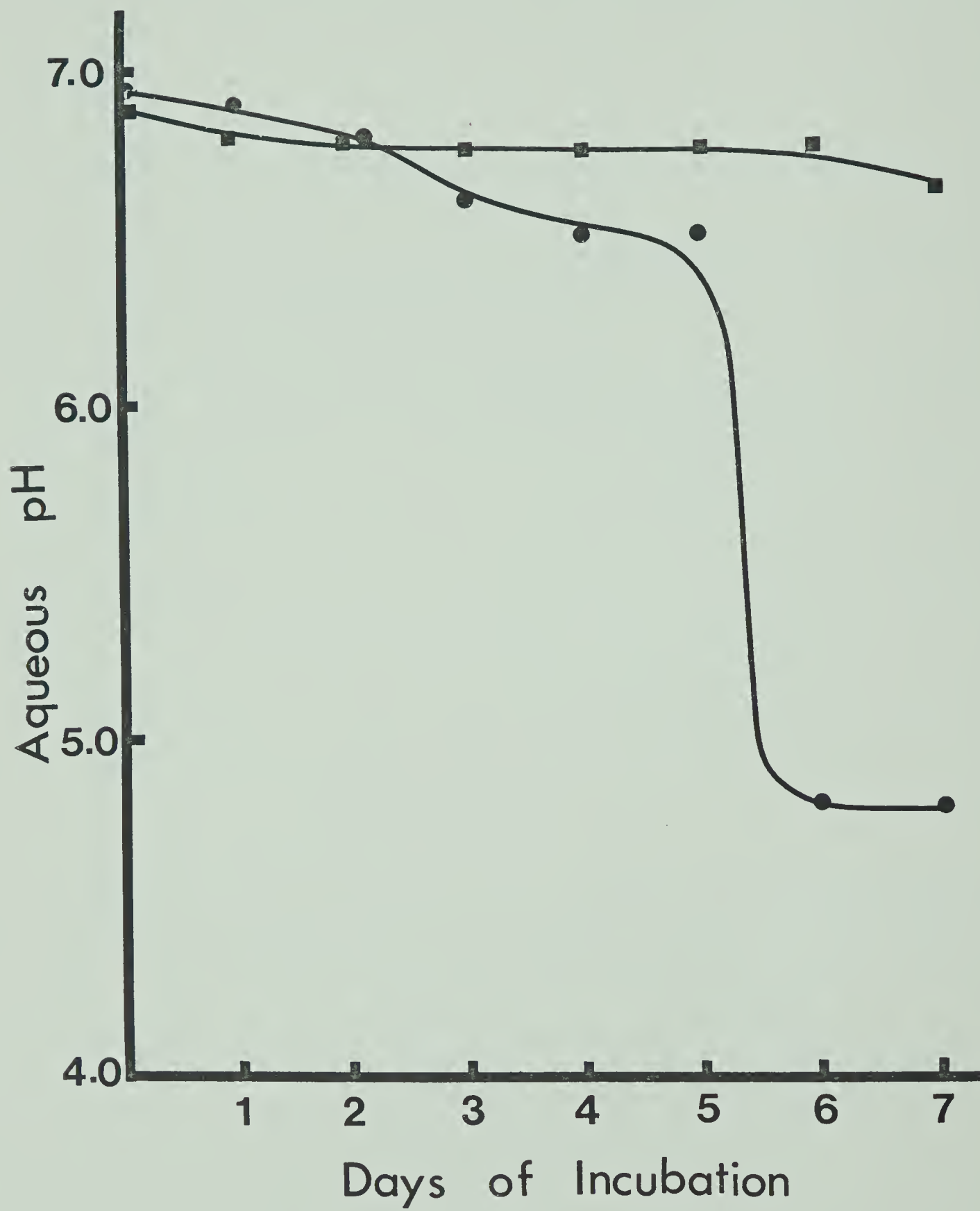


FIGURE 14

pH RESPONSE RESULTING FROM MIXED CULTURE
GROWTH ON RECYCLED PETROLEUM

Aqueous pH values were determined daily with samples collected from the incubation flasks.

Graph: Aqueous pH vs. Days of Incubation.

Flask A: Recycling of petroleum
with original soil
mixture

()

Flask B: Recycling of petroleum
with original soil
mixture + 10%
soil suspension

()

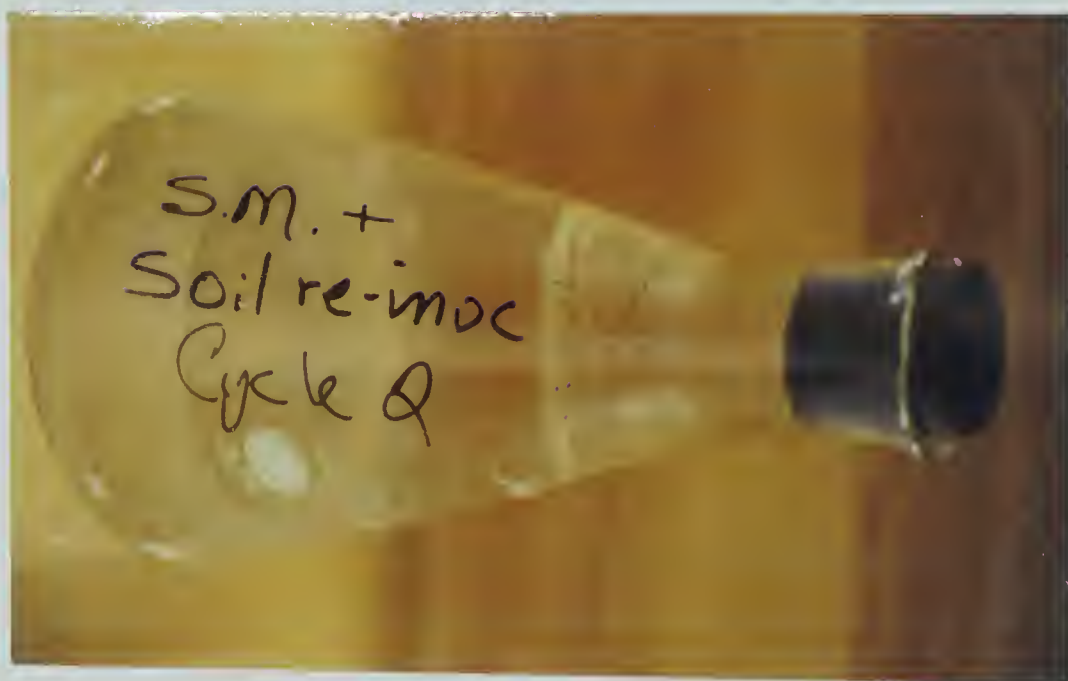
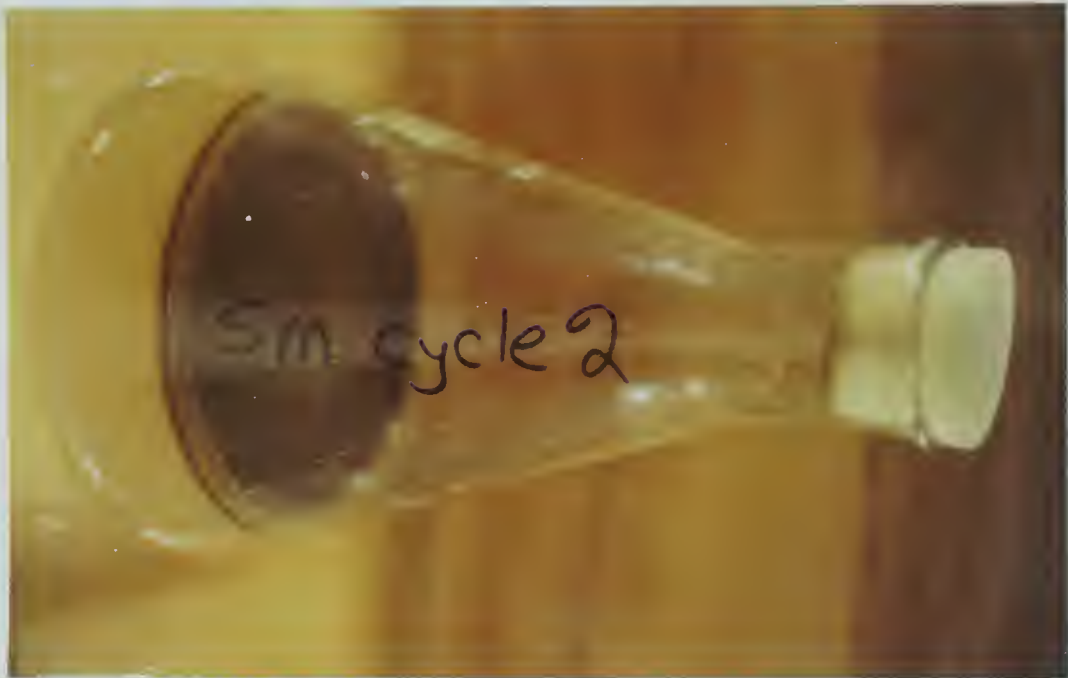


FIGURE 15

APPEARANCE OF SETTLED GROWTH FLASKS AFTER RECYCLING
OF THE RESIDUAL NORTH CANTAL OIL

- Top: Control photograph showing flask with fresh B+N salts + 5.0 mls North Cantal oil immediately after inoculation.
- Middle: Flask A after cycle 2 showing some growth but petroleum still rising to the surface.
- Bottom: Flask B after cycle 2 showing the obvious degradation of the residual petroleum. Deposits at the bottom of the flask are those of the residual petroleum with a specific gravity of greater than one.

initial evidence of further bacterial activity.

That further degradation of the residual petroleum had occurred in cycle 2 in Flask B, was confirmed by liquid and gas chromatographic analyses of the extracted oil (Table IX and Figure 16).

In reviewing the results of this experiment, one is still puzzled by the fact that the original mixed culture was unable to carry out further degradation of the residual oil in cycle 2 even though n-saturate components were still present in appreciable amounts. Whether this original soil mixture required certain unidentified components in the petroleum for growth is not known. If this had been the case, it is possible that these components had already been utilized in cycle 1, thus preventing further growth in Flask A during the second cycle of growth. The possibility of toxic end-product formation does exist, but the diluting effect of aspirating off spent medium and cells and replacing this with fresh B+N salts medium, before cycle 2, should have overcome such a problem.

The utilization of petroleum components in Flask B when fresh soil inoculum was added without any increase in viable counts, also presents a problem of interpretation. It is highly unlikely that a new bacterial population might have been induced which did not appear on B+N counting agar during plate count exercises. Oil utilization, without apparent growth could also be taken to mean that the soil

TABLE IX

LIQUID CHROMATOGRAPHIC ANALYSIS OF RESIDUAL OILS
FROM RECYCLING EXPERIMENT

Component	Residual Oils	
	*Flask A	"Flask B
	Weight %	
Benzene-soluble asphaltenes	3.3	3.8
Benzene-insoluble asphaltenes	5.1	11.8
Saturates	48.8	38.6
Aromatics	32.3	33.5
Soluble NSO	9.5	8.5
Insoluble NSO	0.5	3.5

Topping temperature: 32°.

*Flask A: Recycling of oil with same Christmas tree soil mixture.

"Flask B: Recycling of oil with same soil mixture + 10% added soil suspension.

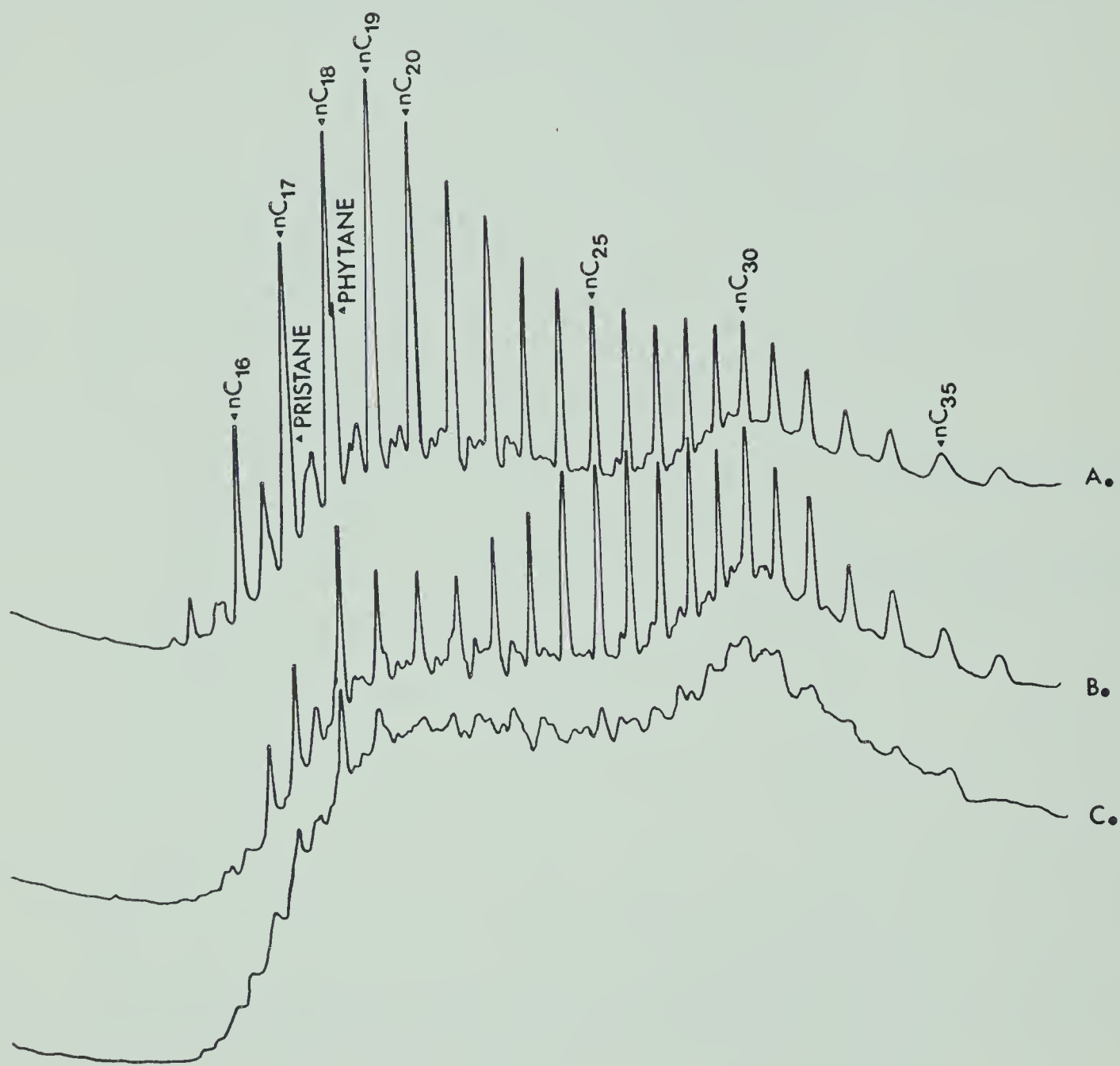


FIGURE 16

GLC PROFILES OF RESIDUAL SATURATE FRACTIONS
FROM RECYCLING EXPERIMENT

- A. North Cantal control saturates pattern.
- B. Saturates from Flask A residual oil after
cycle 2.
- C. Saturates from Flask B residual oil after
cycle 2.

provided certain nutrient(s) to the bacterial population already present (carry-over from cycle 1) which allowed them to further degrade the residual petroleum. To prove the latter theory, it would be necessary to run a control using a 10% soil suspension which was previously autoclaved or otherwise sterilized. In this case, lack of subsequent utilization would indicate the former theory to be correct; while growth and subsequent utilization would mean the soil was acting as a nutrient source for the established soil population remaining from cycle 1. Unfortunately this control was never attempted and lack of time prevented the repeating of the experiment. At any rate, the pH trend indicates that subsequent oil metabolism was occurring in Flask B, and residual analysis bears out this fact.

IX. Growth of the Christmas Tree Soil Mixture on Purified Components of Pre-Metabolized North Cantal Oil

It was shown previously that the christmas tree soil mixture did not have the ability to produce secondary growth on a sample of North Cantal oil after it had initially grown successfully on the same sample. This was irrespective of the fact that the residual oil still showed n-saturate content in liquid and gas chromatographic analysis. Therefore it was decided to metabolize an excess of North Cantal oil with the christmas tree soil mixture; and after recovering

the residual oil, subject it to chromatographic separation to obtain purified components for feeding back to the same soil mixture. By this method, it was hoped that at least three questions would be answered. The first important question was whether the soil mixture would further metabolize the pure residual saturate fraction, since it was known that n-saturates still existed in the sample. The second question centred around whether the soil mixture possessed the biochemical capabilities of metabolizing the other purified components of the residual oil. This would be monitored as an increase in viable counts, if the capabilities existed. The final question, which it was hoped this experiment would answer, had to do with the repeated observation that the soil mixture seemed to always terminate growth in the order of 10^8 viable cells/ml. This occurred whether or not further growth substrate existed within the medium. Although other experiments in the text show a rough linear increase in final counts as a function of amount of oil provided, it always seemed growth stayed within $1 - 5 \times 10^8$ viable cells/ml. It was hoped that by feeding the same soil mixture the petroleum components in pure form, one of the components would perhaps stimulate growth to 10^9 viable cells/ml or even higher.

In beginning the experiment, as mentioned before, metabolized oil was produced by growing the soil mixture in

B + N salts + 5.0 mls North Cantal oil/liter. The flask was agitated at 300 rpm with an incubation temperature of 30° for seven days. At the end of this period the residual oil was extracted with n-pentane from the aqueous medium and fractionated by liquid chromatography. Samples of the purified components were then fed back, separately, to the christmas tree soil mixture. The components were given in the same proportion as they would have been added within a whole oil at the rate of 0.2 gms/200 mls of B + N salts. Thus the benzene-soluble asphaltenes, saturates, aromatics, and soluble NSO components were fed back to the soil mixture at the rate of 0.0988, 0.0055, 0.0624, and 0.0159 gms respectively. This was accomplished by transferring the various components in a solvent, to separate, dry, sterile 500 ml flasks. The solvent was removed by aeration, leaving the pure components coated to the walls of the flask. Two hundred mls of sterile B + N salts was then added to each flask. Prior to incubation, 10 mls of a 5 day culture of the christmas tree soil mixture grown on North Cantal oil was added to each flask. An additional 500 ml flask containing 200 mls of B + N salts medium + 10 mls inoculum was added to the experiment as a control for growth on residual oil carry-over in the inoculum. All flasks were incubated on the same shaker (agitation, 300 rpm) at 30° for seven days. During this time, samples were periodically taken for

purposes of viable counts and aqueous pH determinations. At the end of the incubation period, the flask containing the saturate fraction was n-pentane extracted for collection of the residual saturates. These were passed through a silica gel-alumina gel column to ensure purity, and a GLC profile of the sample was obtained. The results of the viable counts, as presented in Figure 17 indicate that significant growth occurred only on the saturate fraction. All other growth on the remaining components was not significantly different from the growth in the control flask. In addition, growth on the saturates yielded viable counts, not exceeding $1 - 2 \times 10^8$ /ml; thus stimulation of growth over that obtained in whole oils did not occur. It would therefore seem that a more sophisticated control mechanism is at work here, which limits growth, under the present system, to the range of 10^8 viable cells/ml.

The aqueous pH measurements (Table X) indicate that the acidic reaction seen with whole oils can be attributed to aspects of saturates metabolism, since the greatest pH depression occurred in the saturates flask.

The GLC profile, Figure 18, certainly shows that residual n-saturates remained even though growth had apparently ceased. It is also apparent that the soil mixture, when attacking the saturates in a pure form, does so in a manner which differs from the mechanism in whole

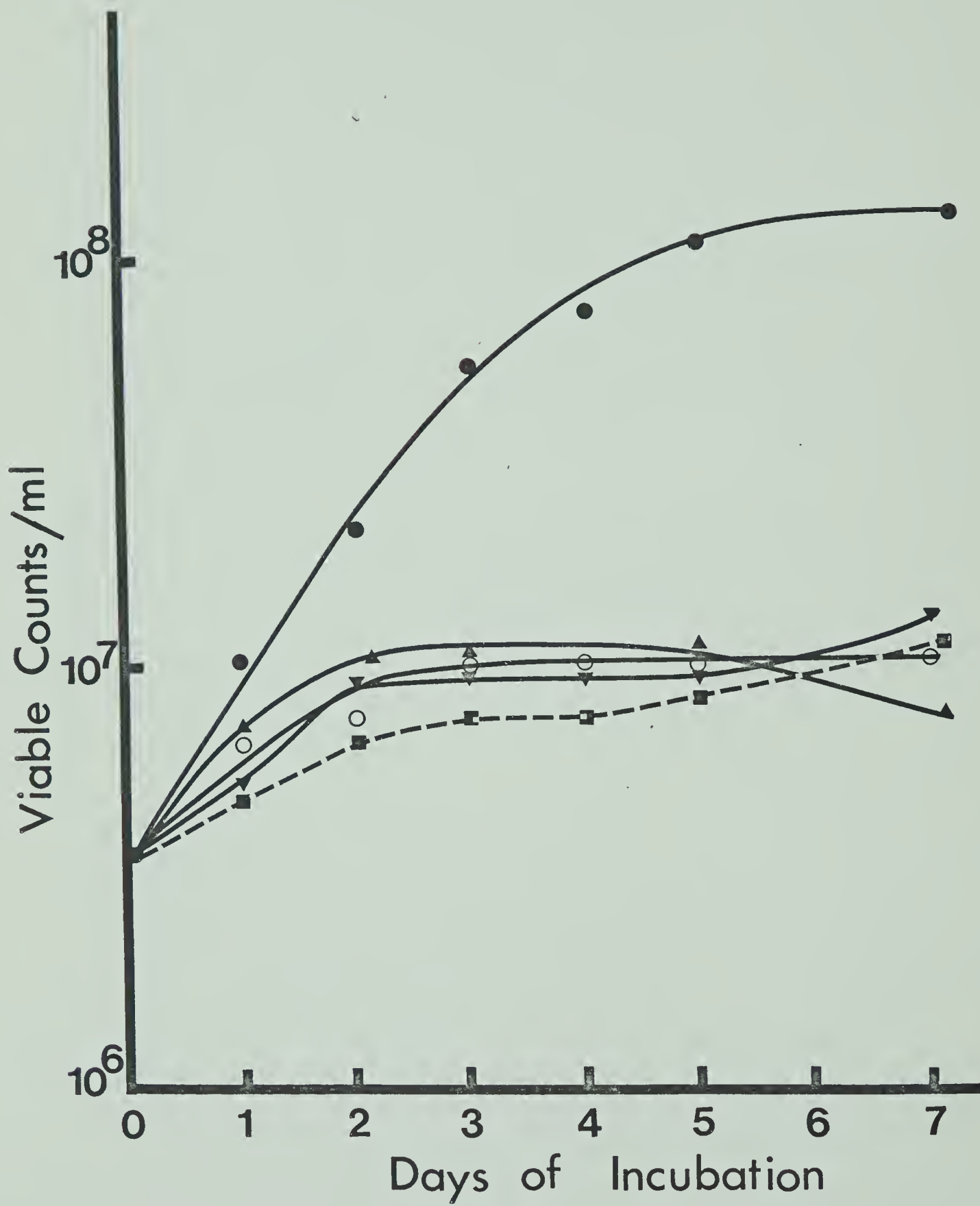


FIGURE 17

GROWTH OF CHRISTMAS TREE SOIL MIXTURE ON
PURIFIED COMPONENTS OF RESIDUAL NORTH CANTAL OIL

Viable counts carried out as described in the text.

Graph: Viable Counts/ml vs. Days of Incubation.

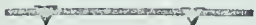

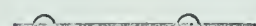

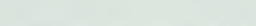
Benzene-soluble asphaltenes:	()
Saturate fraction	()
Aromatic fraction	()
NSO fraction	()
Control growth	()

TABLE X

pH TRENDS FROM CHRISTMAS TREE
 SOIL MIXTURE GROWTH ON VARIOUS PURE
 COMPONENTS FROM RESIDUAL NORTH CANTAL OIL

Day	Growth Component				
	Soluble Asphaltenes	Saturates	Aromatics	NSO	Control
0	6.90	6.90	6.90	6.90	6.90
1	6.80	6.50	6.70	6.85	6.90
2	6.85	6.40	6.70	6.85	6.90
3	6.85	6.35	6.70	6.85	6.85
4	6.85	6.35	6.80	6.85	6.85
5	6.80	6.35	6.75	6.85	6.85
6	6.80	6.30	6.70	6.80	6.80
7	6.80	6.25	6.70	6.80	6.80

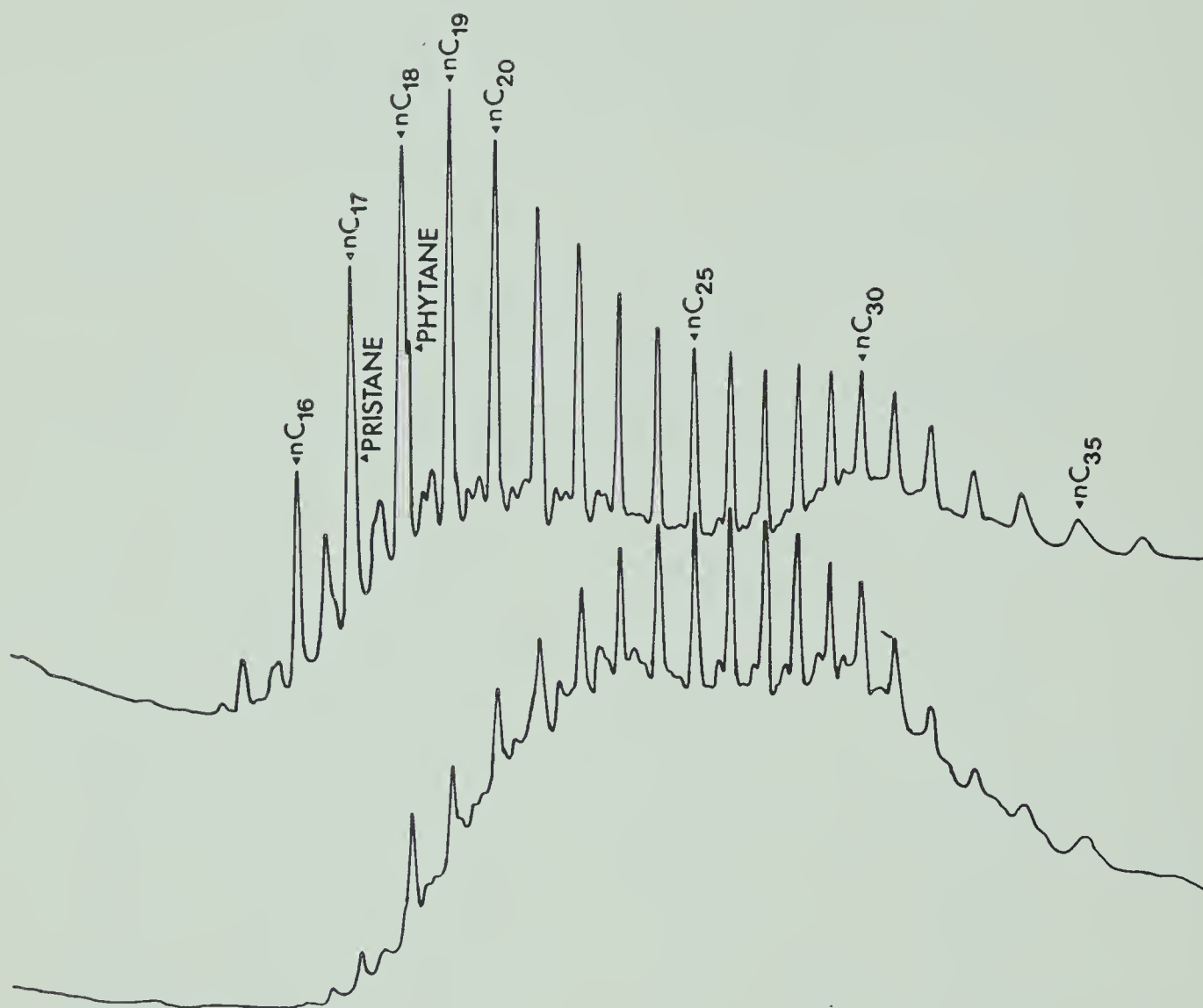


FIGURE 18

GLC PROFILE OF RESIDUAL, PURIFIED SATURATE
COMPONENTS AFTER METABOLISM BY THE
CHRISTMAS TREE SOIL MIXTURE

Top: Profile of North Cantal control saturates.

Bottom: Profile of residual saturates after
serving as a sole carbon substrate for
the Christmas Tree soil mixture.

oil fermentations. When metabolizing a whole oil (refer to Figure 6), the christmas tree soil mixture completely utilizes all n-saturates up to and including nC_{24} . Only when these are exhausted will the organisms then degrade the heavier n-saturates and finally pristane and phytane. When the same soil mixture metabolizes the pure saturate fraction, (Figure 18), it appears the n-saturates are utilized in ascending order starting with the lighter components. Also the pristane and phytane components are utilized in advance of the heavier n-saturates. There is also some indication of partial utilization of the lighter portions of the envelop (isoparaffins, etc.) in advance of the heavier n-saturates. This is shown by the fact that the bulk of the envelope seems skewed to the right when one compares its profile to a North Cantal control saturates profile.

X. Sequential Utilization of North Cantal Oil by a Micrococcus Isolate

It was decided, at this time, to include another single isolate, other than a pseudomonad, in the sequential petroleum utilization study, which would perhaps demonstrate unique growth and degradative capabilities. Among the more than 190 isolates obtained from three soil samples, as described in Materials and Methods, roughly five pure isolates showed promising growth (viable counts), on B+N

salts + North Cantal oil. Four were rejected since they appeared to be pseudomonads and a Pseudomonas species (isolate # 6c(b)100) had already been studied earlier in the investigation. The fifth isolate produced tiny pink, round colonies on B + N agar and microscopic analysis strongly indicated a Micrococcus strain was involved. This tentative identification was accepted for purposes of this investigation, since it was a small Gram (+) coccus. Micrococci are common soil organisms, and numerous reports in the literature indicate that micrococcal isolates have shown abilities to utilize hydrocarbons. This is opposed to the rather fastidious nutrient requirements in Staphylococcus sp. and Streptococcus sp. The other Gram (+) cocci, namely Sarcina and Gaffkya, have never been implicated as being hydrocarbon utilizers.

This isolate was used in a sequential utilization experiment with the North Cantal oil, in much the same manner as described earlier, using the christmas tree soil mixture. Six, two liter flasks, containing one liter of B+N salts + 0.1% North Cantal oil, were inoculated with 20 mls of culture/flask, and incubated for 21 days according to the conditions stipulated in Materials and Methods V. For the first seven days of the experiment, one flask was sampled daily for purposes of pH determination. At days 3, 5, 7, 14, and 21, single flasks were removed from the experiment for n-pentane extraction and chromatographic analyses of the residual

petroleum. Viable count determinations were also carried out during the first seven days of incubation.

The first obvious difference in the growth of the Micrococcus sp. on oil as compared to the Pseudomonas isolate or the christmas tree mixture was in the cell concentration/ml which developed. The viable counts (Figure 19), of the Micrococcus never exceeded 2×10^7 viable cells/ml; while on an equal amount of the same oil, the christmas tree mixture reached a count of $1 - 2 \times 10^8$ viable cells/ml. Whether this difference in counts was made up in the soil mixture by members of the mixture growing on end-products or cell products of the others while not, themselves, attacking the petroleum, is not known. Nevertheless, repeated experiments with the Micrococcus showed its upper limits of growth on B + N salts was in the order of 10^7 cells/ml. Therefore, it seems that an unknown control mechanism also exists in this organism which dictates upper limits of growth in this system.

pH measurements (Figure 20) indicated, as with the soil mixture, that growth of the Micrococcus sp. on oil as a sole carbon substrate yielded an acidic reaction.

Liquid chromatographic analyses, the results of which are presented in Table XI, show that while the isolate produced only roughly one-seventh the number of viable counts as did the soil mixture, it was capable of accomplishing nearly as much saturates degradation. Thus one could say

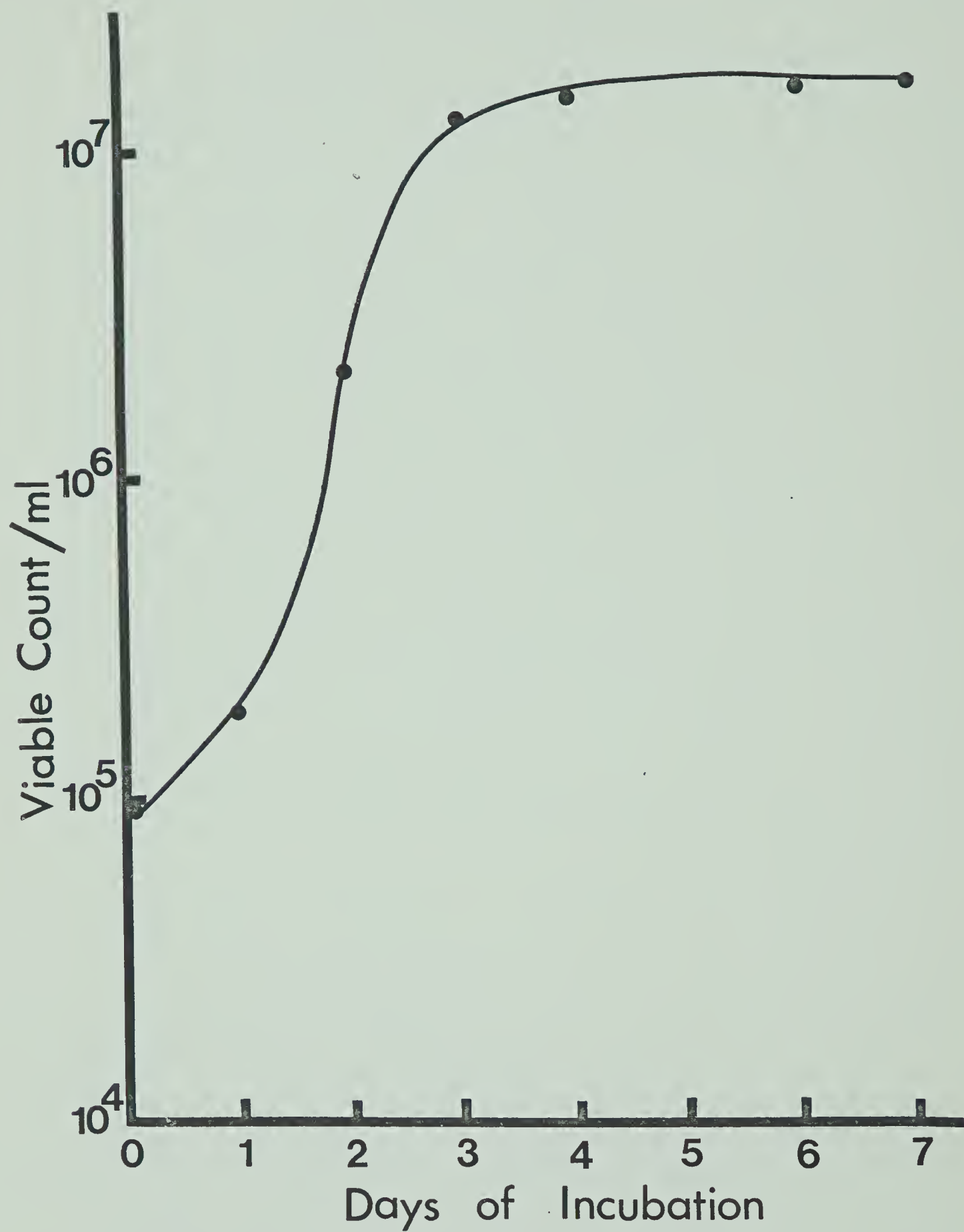


FIGURE 19

GROWTH OF A MICROCOCCUS ISOLATE ON B+N SALTS
MEDIUM + 0.1% NORTH CANTAL OIL

Method of viable count is as presented in the text. Plates were incubated for seven days at 30° before counts were made.

Graph: Viable count/ml vs. Days of Incubation.

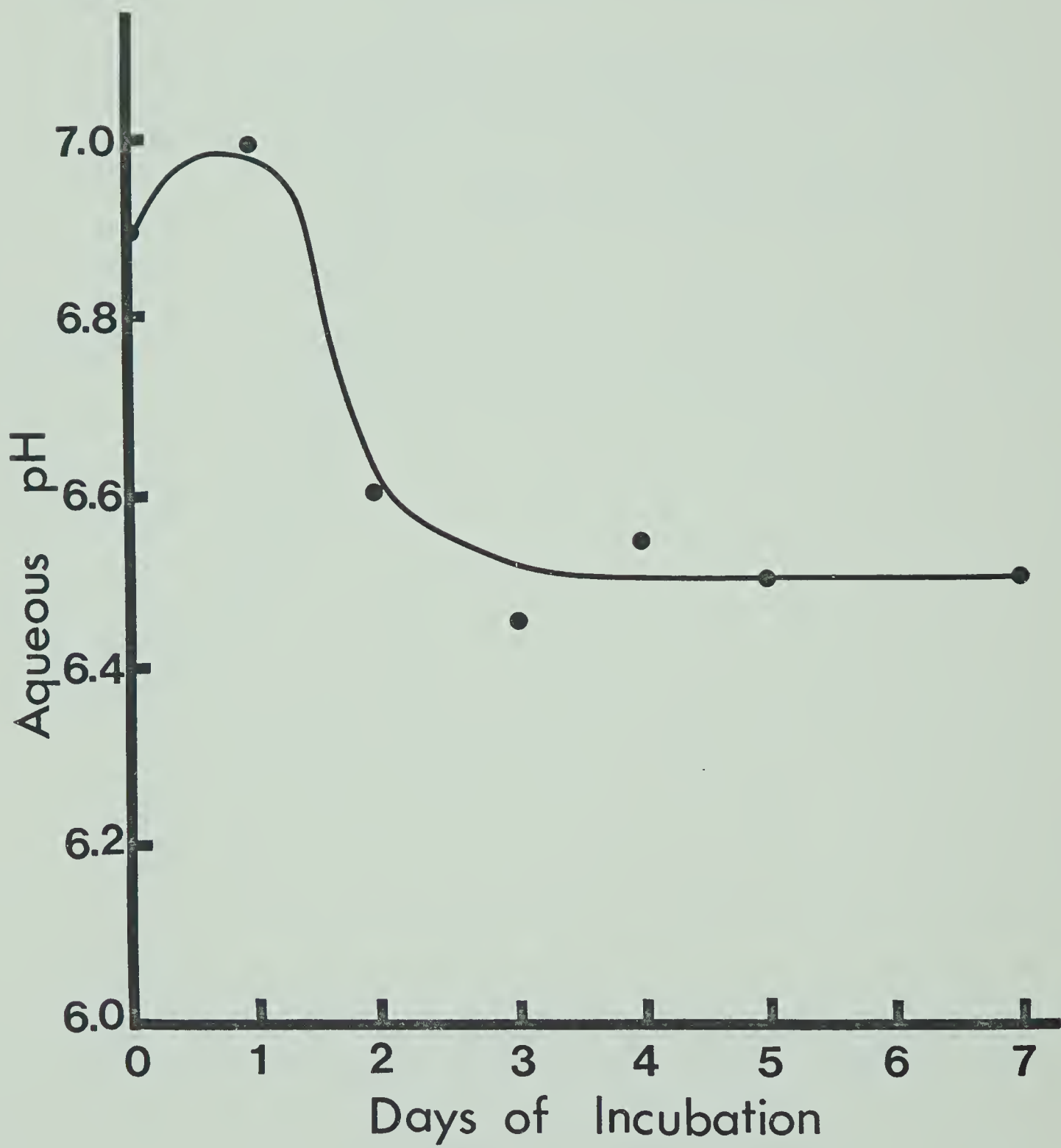


FIGURE 20

pH CHANGES DURING GROWTH OF THE MICROCOCCUS
ISOLATE ON B+N SALTS + 0.1% NORTH CANTAL OIL

Aqueous pH measurements were carried out as
described earlier in the text.

Graph: Aqueous pH vs. Days of Incubation.

TABLE XI

LIQUID CHROMATOGRAPHIC ANALYSIS OF RESIDUAL PETROLEUM
FOLLOWING SEQUENTIAL UTILIZATION BY THE MICROCOCCLUS ISOLATE

Component	Day of Sampling					Weight %
	2	3	5	7	14	21
Benzene-soluble asphaltenes	4.0	4.7	3.9	4.4	4.9	3.9
Benzene-insoluble asphaltenes	5.8	5.3	8.3	6.3	5.1	5.5
Saturates	51.0	40.9	37.0	37.8	36.6	38.0
Aromatics	31.0	32.9	32.5	33.1	35.7	36.15
Soluble NSO	6.1	9.2	10.2	10.4	11.7	11.2
Insoluble NSO	0.5	6.8	6.9	7.8	5.7	4.9

All values expressed as the weight percentage of the topped weight of oil.

Topping temperature: 36.5°.

that the Micrococcus sp. either metabolized oil components at a greatly accelerated rate, or that it used products from the metabolism much less efficiently, therefore requiring more initial petroleum substrate.

Gas chromatographic analyses of the saturates samples obtained by liquid chromatography are presented in Figure 21. Again, comparing these profiles with those obtained from the earlier soil mixture (Figure 6), certain facts become apparent. The Micrococcus sp. attacks all lengths of n-saturates with equal facility. Like the soil mixture, it finds difficulty in utilizing the pristane and phytane components of the saturate fraction; and it only accomplishes this in the later stages of the fermentation.

Liquid and gas chromatographic analysis definitely shows saturates utilization by the Micrococcus isolate. Theoretical enrichment calculations were carried out, based on day 14 analysis, to determine whether other components were in fact being utilized or were being enriched at a rate higher than predicted. Results of these calculations are shown in Table XII. The basis for the theoretical calculations are the same as for the results presented before in Table VII. These calculations of theoretical enrichment, when compared to actual Day 14 analyses, seem to indicate that the insoluble asphaltenic and NSO character is added to by amounts greater than would be expected. This apparently means that the Micrococcus sp. produces small

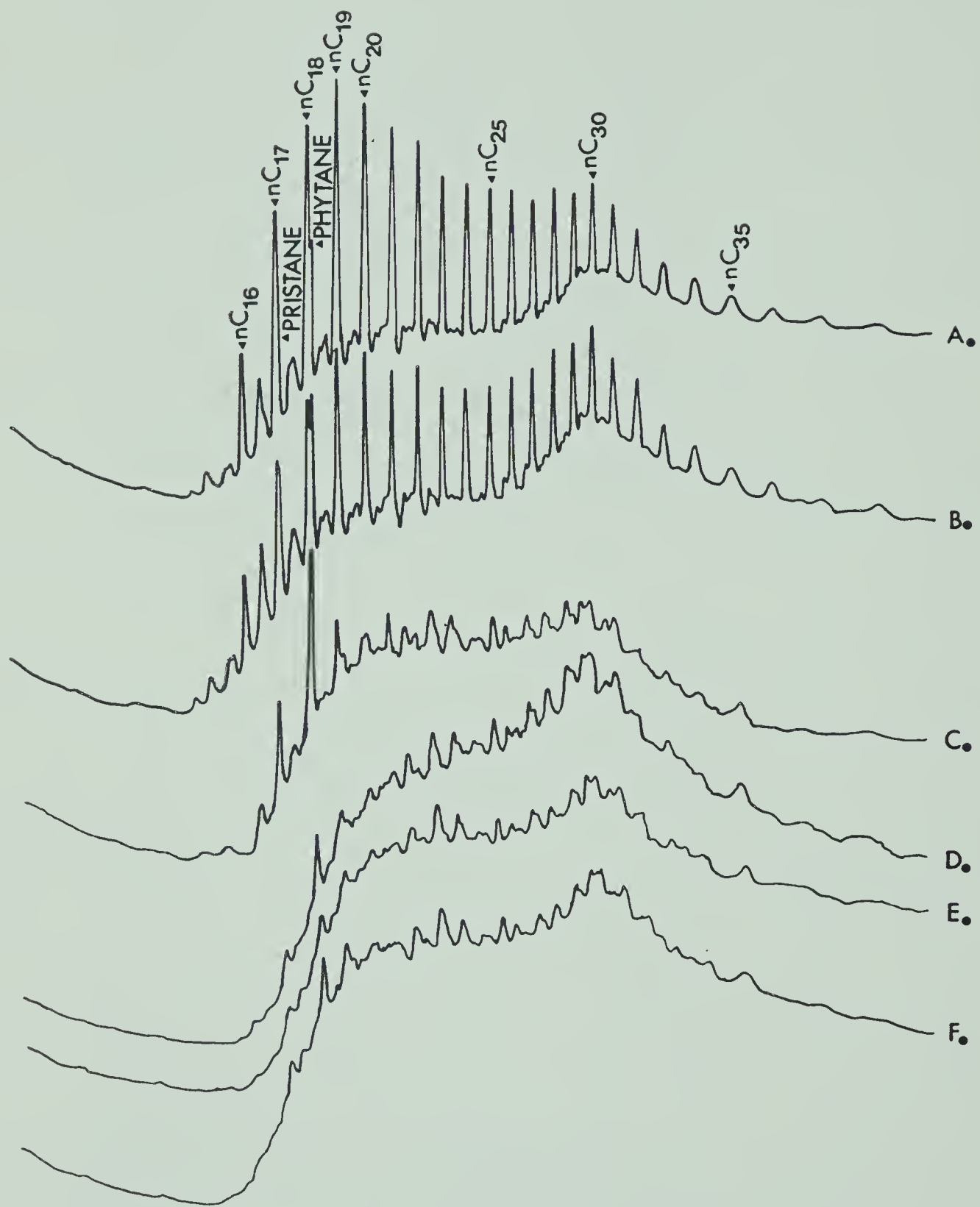


FIGURE 21

GAS CHROMATOGRAPHIC ANALYSES OF RESIDUAL
SATURATES FOLLOWING SEQUENTIAL UTILIZATION
BY THE MICROCOCCUS ISOLATE

- A. Original North Cantal saturates.
- B. Saturates recovered after day 2 of incubation.
- C. Saturates after day 5 of incubation.
- D. Saturates after day 7 of incubation.
- E. Saturates after day 14 of incubation.
- F. Saturates after day 21 of incubation.

TABLE XII

ACTUAL AND THEORETICAL ENRICHMENT OR DEPLETION OF PETROLEUM
COMPONENTS FOLLOWING GROWTH OF THE MICROCOCCLUS ISOLATE

Component	Control Cantal oil	Actual Day 14 Analysis	Theoretical Day 14 Analysis
Weight %			
Benzene-soluble asphaltenes	2.5	4.9	3.2
Benzene-insoluble asphaltenes	6.8	5.1	8.7
Saturates	51.1	36.6	36.6
Aromatics	31.2	35.7	40.4
Soluble NSO	8.6	11.7	11.1
Insoluble NSO	0.3	5.7	1.1

Note: The theoretical calculations assume only the saturate fraction was utilized and all other components were negatively enriched.

amounts of end products with these properties. The soluble NSO component is present in the same amounts as predicted indicating its refractory character and lack of biological occurrence. The aromatic fraction definitely shows partial utilization; and the soluble asphaltenes show perhaps some evidence of degradation. Therefore, it would seem that the saturate and the aromatic fractions are both susceptible to attack by the Micrococcus isolate.

XI. Studies on The Sequential Utilization of North Cantal Oil by Non-Obligate Psychrophilic Soil Mixtures

As indicated in the Introduction, experiments were undertaken to determine the ability of microorganisms to metabolize and grow on petroleum at psychrophilic temperatures. The extrapolation of these experiments to problems of waste and spilled petroleum disposal in temperate climates where soil and water temperatures seldom exceed 4 - 5° is of obvious interest.

The isolation methods utilized to develop psychrophilic organisms with petroleum-degrading capabilities have been previously described in Materials and Methods III.

The two mixtures of organisms obtained, termed the christmas tree and Salmon Arm psychrophilic mixtures were grown on B+N agar at 4°, in order to study colony morphology and to provide samples for microscopic analysis.

Each psychrophilic mixture appeared to be composed of three distinct morphological types. In the christmas tree

psychrophilic mixture, macro- and microscopic examination revealed the following:

- a. A small Gram (-) rod which produced rough-edged, flat, yellow colonies. It was tentatively classified as a Pseudomonas or Xanthomonas strain.
- b. A small, thick Gram (-) rod which appeared to possess a muccopolysaccharide sheath. On a plate, it appeared as large, creamy-white globose colonies of ropey consistency. No attempt was made to classify the organism.
- c. A fairly large Gram (+) rod with no apparent spores. It appeared as creamy-yellow, raised colonies on agar. Because of its Gram reaction, it was tentatively classified as a Bacillus species.

In the Salmon Arm psychrophilic mixture, the organisms appeared as follows:

- a. A small, thick Gram (-) rod, exactly the same as described in (b.) of the christmas tree psychrophilic mixture.
- b. A small Gram (-) rod, producing creamy, round, smooth-edged colonies. It was tentatively classified as a Pseudomonas strain.
- c. A Gram (variable) rod exhibiting an abundance of subterminal spores. On agar, it appeared as very small, raised, white colonies. It was tentatively classified as a Bacillus strain.

All isolates from the two mixtures showed growth at 30°, on agar plates, within 72 hours, as well as visible growth within 10 days at 4°.

Both the psychrophilic mixtures were used in separate, sequential utilization experiments. The design of these experiments was as described in Materials and Methods V. In each case, five, two liter flasks, containing one liter of B+N salts, 0.1% petroleum, and inoculum, were incubated at 4°, agitation 280 rpm. At days 3, 5, 7, 14, and 21 of the experiments, one flask from each of the mixtures was n-pentane extracted.

The results of the viable count exercises carried out with both mixtures (Figure 22) seem to indicate the christmas tree psychrophilic mixture is slightly superior in terms of its upper limits of growth; although both cultures exhibit the same length of lag phase. pH measurements (Figure 23) taken during the course of the incubation, however, establish no clear differences between the two cultures. Neither is there any clear evidence of differences existing between the two cultures, in terms of their degradative capabilities, as determined by liquid chromatographic analyses of the residual petroleum samples, (Table XIII). In this case, the christmas tree psychrophilic mixture appears slightly slower in its initial utilization of the saturate components as compared with the Salmon Arm mixture. However, after day 5 of the incubation, no clear differences can be pointed out

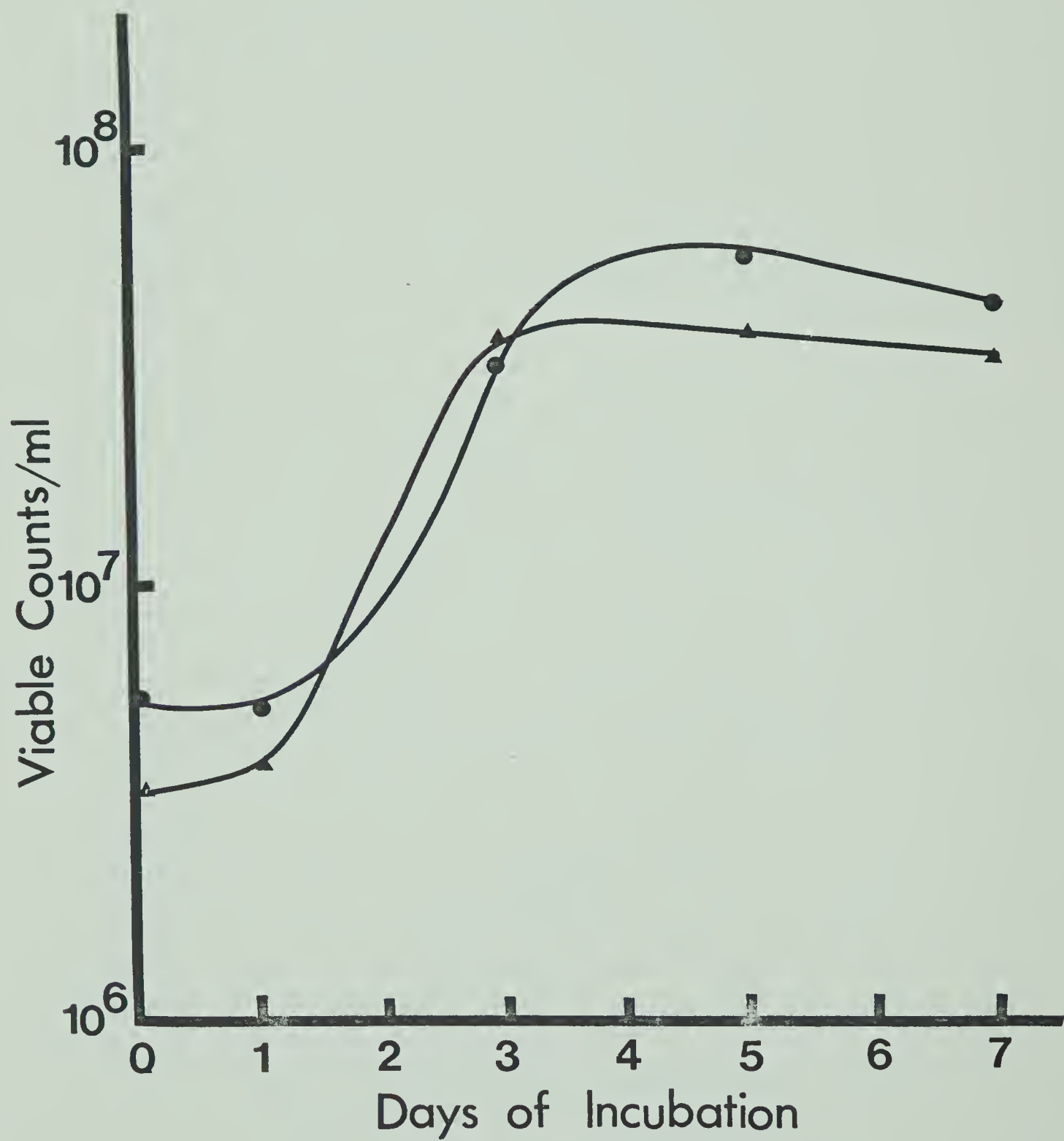

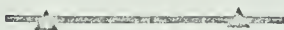


FIGURE 22

GROWTH OF TWO NON-OBLIGATE PSYCHROPHILIC
SOIL MIXTURES GROWING ON B+N SALTS + NORTH CANTAL OIL .

Method of enumeration of viable cells was as
explained earlier in the text.

Graph: Viable cell counts/ml vs. Days of Incubation.

Christmas tree psychrophilic mixture ()
Salmon Arm psychrophilic mixture ()

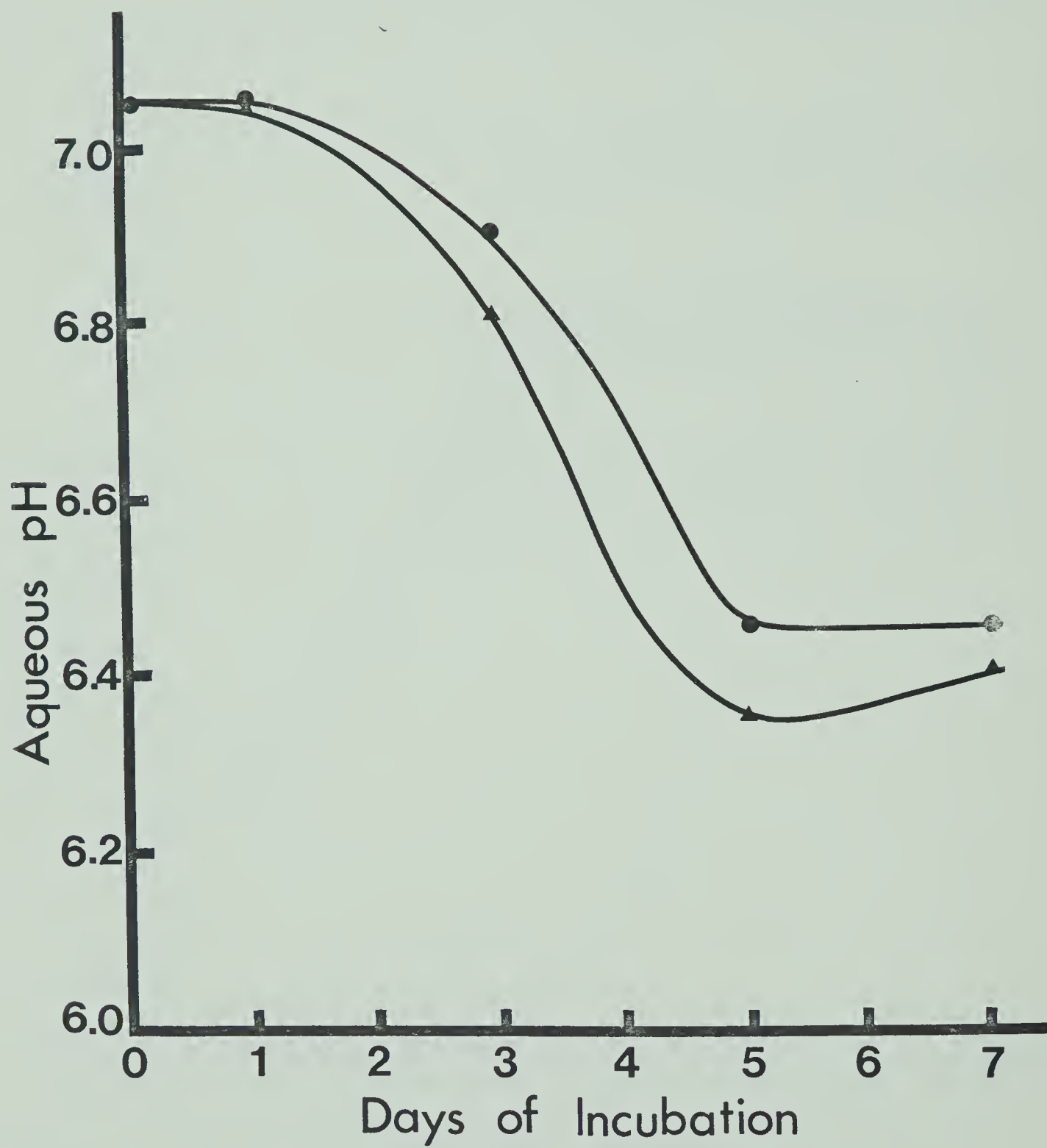


FIGURE 23

pH CHANGES OF NON-OBLIGATE PSYCHROPHILIC MIXTURES
GROWING ON B+N SALTS + 0.1% NORTH CANTAL OIL

pH determinations were made on 1.0 ml samples of whole culture removed from the incubation flasks, and allowed to come to room temperature.

Graph: Aqueous pH vs. Days of Incubation.

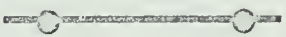

Christmas tree psychrophilic mixture ()
Salmon Arm psychrophilic mixture ()

TABLE XIII

LIQUID CHROMATOGRAPHIC ANALYSES OF RESIDUAL OILS OBTAINED FROM NON-OBLIGATE
PSYCHROPHILIC MIXTURES GROWING ON B+N SALTS + 0.1% NORTH CANTAL OIL

Component	Christmas tree					Salmon Arm				
	(day of sampling)					(day of sampling)				
	3	5	7	14	21	3	5	7	14	21
	Weight %					Weight %				
Benzene-soluble asphaltenes	3.2	3.1	3.5	3.6	3.6	3.4	3.4	3.1	3.8	3.7
Benzene-insoluble asphaltenes	9.3	7.3	7.1	10.3	7.4	11.0	4.5	4.2	5.1	4.8
Saturates	45.2	44.3	39.0	36.8	38.0	40.1	41.6	41.1	40.6	39.5
Aromatics	32.8	34.8	35.2	34.8	37.1	32.7	36.5	36.7	37.5	37.2
Soluble NSO	9.3	10.4	9.9	10.2	11.2	9.4	11.9	11.9	12.1	12.6
Insoluble NSO	0.1	0.1	5.02	4.2	1.4	3.2	2.5	2.7	0.8	2.2

Topping temperature: 32°.

which would indicate any advantages of one of the mixtures over the other. Figures 24 and 25, showing GLC profiles of residual saturate samples from the two fermentations, indicate a similarity in degradative behavior of the two psychrophilic mixtures. Figure 26 shows photographs of the day 21 flasks of both fermentations after they were allowed to stand for four hours at room temperature. The residual oils from both flasks show specific gravities greater than 1.0 since both samples have settled to the bottom of the flasks. Pigment production is apparent in both aqueous phases. The nature of these pigments was not investigated.

The general conclusion which can be drawn from this experiment is that psychrophilic microorganisms certainly have the ability to degrade at least certain components from a whole petroleum provided as a sole carbon substrate. In their attack on the n-saturates within the crude oil, the christmas tree psychrophilic mixture appears to utilize all n-saturates with equal facility up to but not including hentriacontane and above, (see profile B, Figure 23). These saturates from nC_{31} onward are utilized on further incubation, by day seven. It is rather strange that the psychrophilic mixture could use n-saturates without discrimination up to this chain length, at 4°; while a mesophilic soil mixture showed temporary reluctance to use saturates from penta-cosane (nC_{25}) onward. Thus, factors other than solubilities of components at a given temperature must enter into the

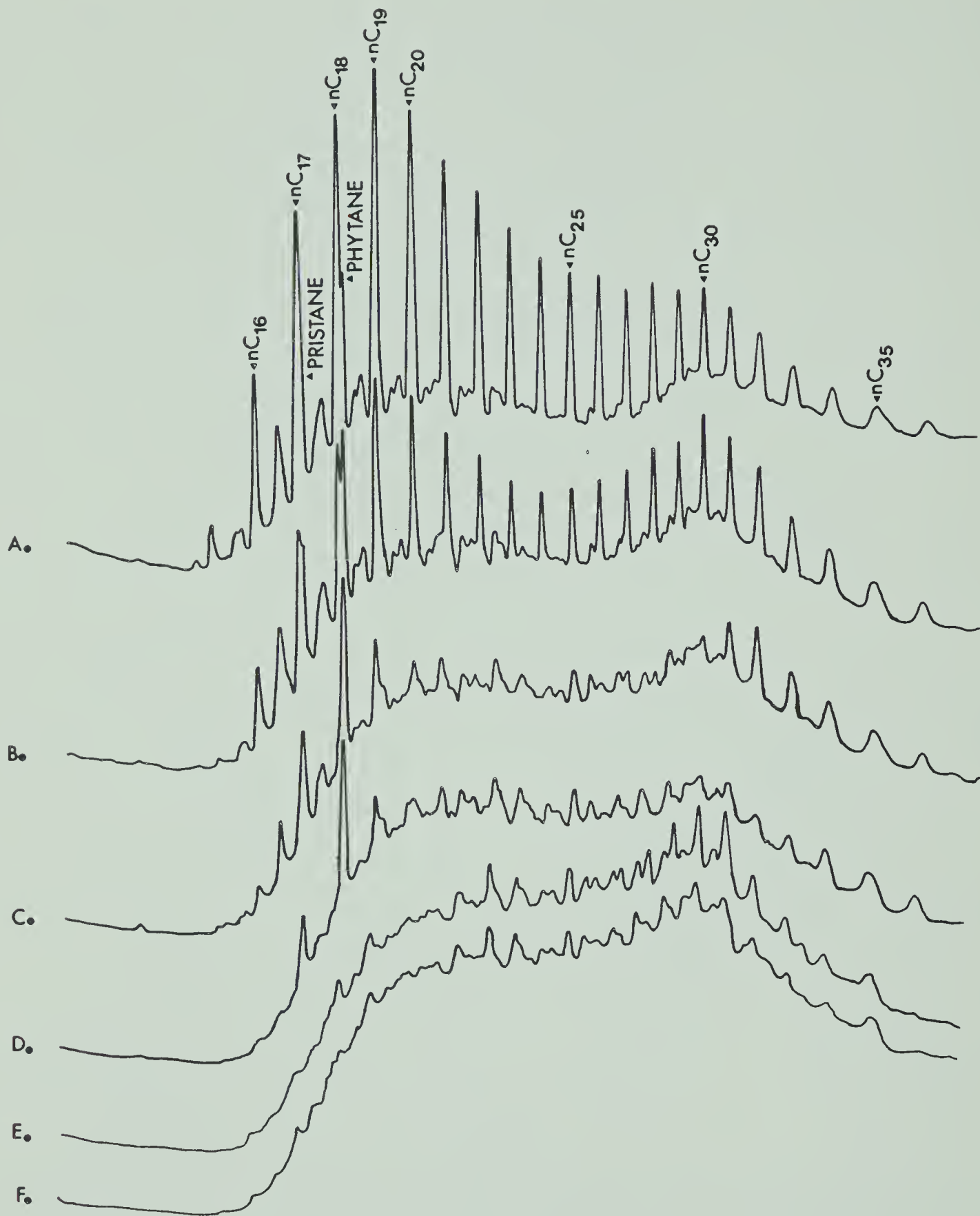


FIGURE 24

SEQUENTIAL RESIDUAL SATURATES PROFILES FROM GROWTH
STUDY OF CHRISTMAS TREE PSYCHROPHILIC MIXTURE

- A. GLC profile of North Cantal control saturates.
- B. GLC profile of Day 3 residual saturates.
- C. GLC profile of Day 5 residual saturates.
- D. GLC profile of Day 7 residual saturates.
- E. GLC profile of Day 14 residual saturates.
- F. GLC profile of Day 21 residual saturates.

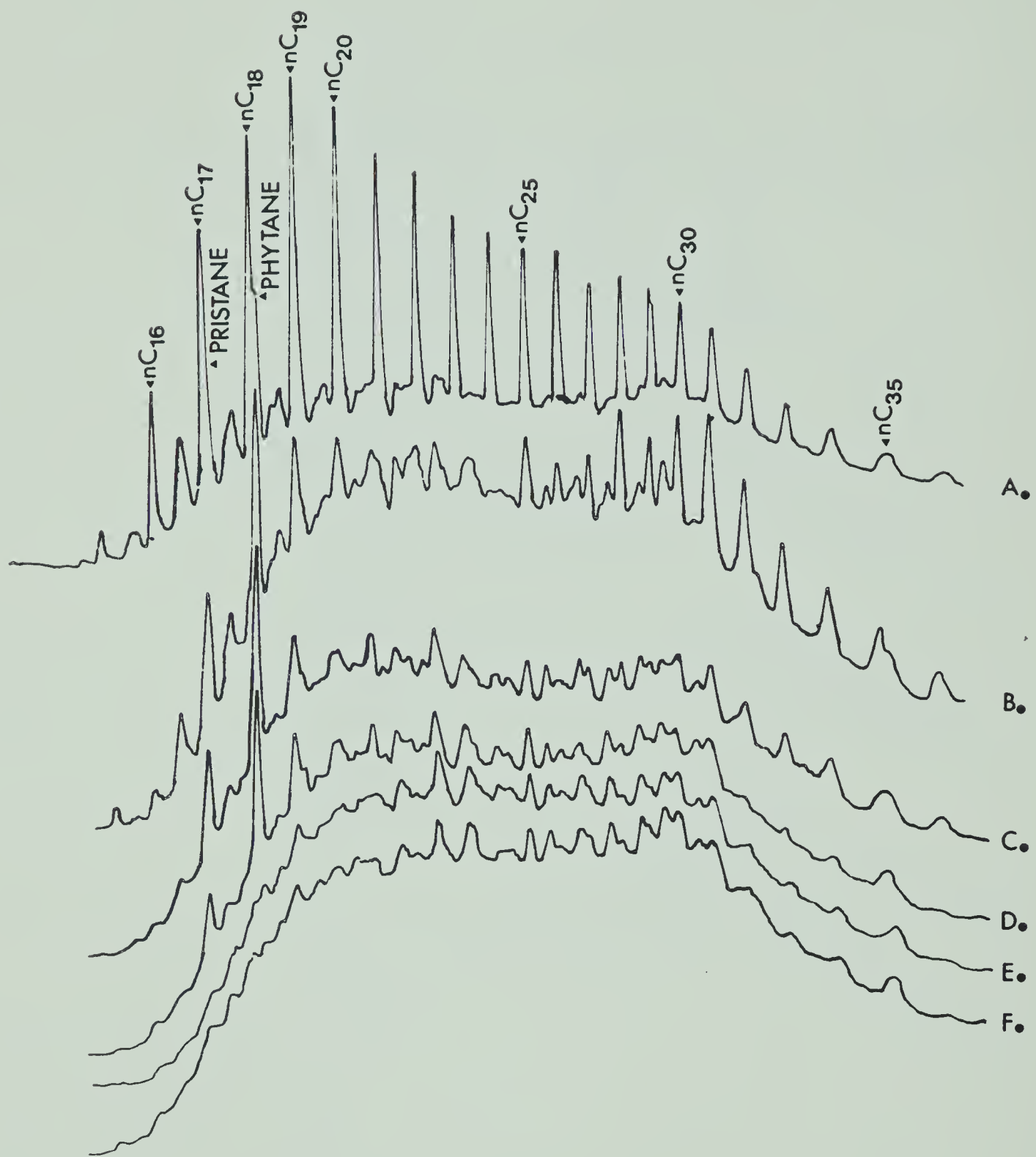


FIGURE 25

SEQUENTIAL RESIDUAL SATURATES PROFILES FROM GROWTH
STUDY OF SALMON ARM PSYCHROPHILIC MIXTURE

- A. GLC profile of North Cantal control saturates.
- B. GLC profile of Day 3 residual saturates.
- C. GLC profile of Day 5 residual saturates.
- D. GLC profile of Day 7 residual saturates.
- E. GLC profile of Day 14 residual saturates.
- F. GLC profile of Day 21 residual saturates.



FIGURE 26

PHOTOGRAPHS SHOWING APPEARANCE OF GROWTH OF NON-
OBLIGATE PSYCHROPHILES ON NORTH CANTAL OIL
AFTER 21 DAYS INCUBATION

Top: Results of Salmon Arm psychrophilic mixture
growing on North Cantal oil for 21 days.

Bottom: Results of christmas tree psychrophilic mixture
growing on North Cantal oil for 21 days.

Both flasks were allowed to stand for 4 hours before
photographs were taken.

Note: In both flasks, the residual oil remains precipitated
indicating a specific gravity of greater than 1.0.

question of n-alkane uptake by bacteria. In this respect, the Salmon Arm psychrophilic mixture differs slightly from the christmas tree psychrophiles. While the christmas tree mixture showed temporary discrimination against nC_{31} and beyond, the Salmon Arm mixture showed a similar temporary block from octacosane (nC_{28}) onward, (see Figure 24, Profile B).

Theoretical calculations were made, using the day 21 chromatographic data from both psychrophilic mixtures, to determine whether utilization or production of oil components other than the saturate fraction had occurred. The results of the calculations are presented in Table XIV.

The results shown in Table XIV seem to indicate that neither psychrophilic mixture is capable of significant alteration on components other than the saturate fraction. It would appear that the christmas tree and perhaps the Salmon Arm psychrophilic mixtures show activity against the aromatic fraction. Neither the benzene-soluble asphaltenes nor the soluble NSO's are significantly different from the amounts predicted by negative enrichment. There is slight evidence indicating solubilization of small amounts of the insoluble asphaltenes and production of insoluble NSO's. Thus, in general, one must conclude that either the psychrophiles are not as diverse in their abilities as are the mesophiles; or that utilization of non-saturate components at psychrophilic temperatures takes a great deal longer to accomplish. Nevertheless, it does prove that the non-saturate character

TABLE XIV

COMPARISONS BETWEEN CALCULATED AND ACTUAL ENRICHMENTS OF NON-SATURATE COMPONENTS
BY THE CHRISTMAS TREE AND SALMON ARM PSYCHROPHILIC MIXTURES

Component	North Cantal control	Actual day 21 analysis	Theoretical day 21 analysis	Actual day 21 analysis	Theoretical day 21 analysis
Benzene-soluble asphaltenes	2.5	3.6	3.3	3.7	3.2
Benz ne-insoluble	6.8	7.4	8.4	5.8	7.9
Saturates	51.1	38.0	38.0	39.5	39.5
Aromatics	31.2	37.1	39.0	37.2	38.2
Soluble NSO	8.6	11.2	11.0	12.6	11.0
Insoluble NSO	0.3	1.4	0.6	2.2	0.7

All calculations (theoretical) are based on the assumption that only saturates utilization occurred, yielding negative enrichment of all other components.

of oil will be difficult to be disposed of, biologically, under cold temperature conditions.

XII. Preliminary Study Concerning the Degradation of Lost Horse Hill Oil - an Inferior Oil of the Deposit

As stated earlier in the literature review, the oil fields of interest to Imperial Oil Ltd. in Saskatchewan, range from the superior North Cantal type oil in the south-eastern portion of one field to the inferior Lost Horse Hill oil in the north-west sector. Also, as mentioned previously, it is theorized that a bacterial process has in fact been responsible for this degradation; hence the basic impetus for this whole investigation. An immediate problem arises however when one fully investigates Lost Horse Hill oil. Although the $^{32}\text{S} : ^{34}\text{S}$ ratios of deposit samples from this area indicate biological fractionation not seen in south-eastern regions of the field; and the liquid chromatographic analysis show definite saturates loss, GLC profiles of the saturates show an almost normal distribution (Figure 28, A). This would almost suggest that Lost Horse Hill oil has, at some time in the past, been diluted with mixtures of aromatics NSO's and asphaltenes, containing no saturates whatever. Thus, an oil with saturates severely diluted, but still maintaining an intact profile would be produced. To propose a geochemical or biological process which could accomplish this is indeed difficult. Fortunately, however,

other producing wells very close to the Lost Horse Hill site show oils also depleted in saturates (35% - 36%) of the topped weight, and these oils do show severe losses of n-paraffins in the GLC profile. An example of such an oil is High Prairie oil. GLC profiles of the saturate fraction from this oil are compared with those of an oil from the opposite end of the field, very comparable to North Cantal. The GLC profiles of the High Prairie sample and the superior North Cantal-related oil sample are presented in Figure 27. The profiles appear different from those shown previously in the text. This is due to the fact that the latter two samples were run on a W 98 column, which presents fewer envelope problems but less resolution of the higher chain-length saturates.

It is obvious that the High Prairie sample shows degradation very similar to that obtained earlier in the text by bacterial metabolism of North Cantal petroleum.

In light of the situation just described, and as a final experiment in the investigation, Lost Horse Hill oil was used as a test substrate for the christmas tree mesophilic soil mixture. It was hoped, first of all, that bacterial metabolism, if it did occur, would yield a residual oil possessing GLC profile properties more closely resembling High Prairie oil, or the previously microbiologically-degraded North Cantal oil. If this could be accomplished, then the investigation would have shown conclusively that



FIGURE 27

GLC PROFILES OF SATURATES FROM A NORTH CANTAL-
RELATED PETROLEUM AND FROM HIGH PRAIRIE PETROLEUM

- A. *Saturates profile of High Prairie oil.
- B. *Saturates of a North Cantal-related oil

Note: These saturates were run on a W98 column; hence
the visible differences in the profiles as compared
to the ones shown previously.

Also note that pristane and phytane, rather than immediately
following nC_{17} and nC_{18} respectively, as in an OV-1 profile,
precede nC_{17} and nC_{18} on a W98 column.

*The Figure is presented with the permission of Imperial
Oil, Producing Department, Calgary.

bacterial action, under aerobic conditions, could satisfy the requirements of the initial hypothesis. In other words, all the varied oils within these Saskatchewan deposits could be reduced in quality, via a microbial process(es) to yield a common petroleum with properties matching or approaching that of the High Prairie oil.

A simple growth trial was carried out, using Lost Horse Hill oil as growth substrate, and the mesophilic christmas tree soil mixture as inoculum. In conjunction with this, the experiment was also run using whole christmas tree soil as inoculum. The reason for this was to perhaps enrich a more efficient population for growth on the less desirable oil. It was thought that the established, initial christmas tree mesophilic mixture might not adapt to this lower quality oil, after growing on Cantal oil for so many generations. Thus four, two liter flasks each containing 1 liter of B+N salts + 0.1% Lost Horse Hill oil were prepared. Two flasks were inoculated with 20 ml portions of a 4 day culture of the established mesophilic christmas tree soil mixture. The other two flasks received 50 ml portions of a 10% preparation of the neat christmas tree soil. Incubation was carried out according to Materials and Methods V (for all mesophilic experiments). No viable count or pH measurements were made, since the liquid and gas chromatographic analyses were the only data of interest at this point.

After 7 days of incubation, all flasks were n-pentane

extracted, and the residual oils subjected to analyses. Table XV shows the results of the liquid chromatographic analysis. It is rather obvious that the established mesophilic soil mixture could not adapt to the different, inferior oil and only small decreases were imparted upon the saturate fraction. On the other hand, the soil enrichment method caused a population of bacteria to be enriched which could degrade the already inferior Lost Horse Hill oil. The GLC profiles of the residual saturate samples (Figure 28, B + C), only further illustrates the results of the liquid chromatographic analysis.

This experiment therefore served as a final proof that the complete spectrum of oils in these Saskatchewan deposits could be metabolized to an n-saturate-depleted residual product. This common residual product matched very closely, the properties of the most degraded petroleum found naturally-occurring in the field, namely the High Prairie oil.

TABLE XV

LIQUID CHROMATOGRAPHIC ANALYSES OF THE RESIDUAL LOST HORSE HILL
OIL SAMPLES FOLLOWING BACTERIAL METABOLISM

Component	Lost Horse Hill Control				Mesophilic christmas tree soil mixture		Neat soil enrichment	
	Sample A.	Sample B.	Sample A.	Sample B.	Sample A.	Sample B.	Sample A.	Sample B.
	Weight %							
Benzene-soluble asphaltenes	7.8		7.7	9.0		9.2		12.8
Benzene-insoluble asphaltenes	9.9		5.1	4.8		12.2		8.1
Saturates	36.8		29.7	29.3		17.8		18.7
Aromatics	37.9		38.7	38.4		40.3		40.1
Soluble NSO	13.1		16.8	17.4		15.4		16.6
Insoluble NSO	0.2		1.9	0.9		5.6		3.5

All values are expressed as weight percentages of the topped weights of oil.

Topping temperature: 32°.

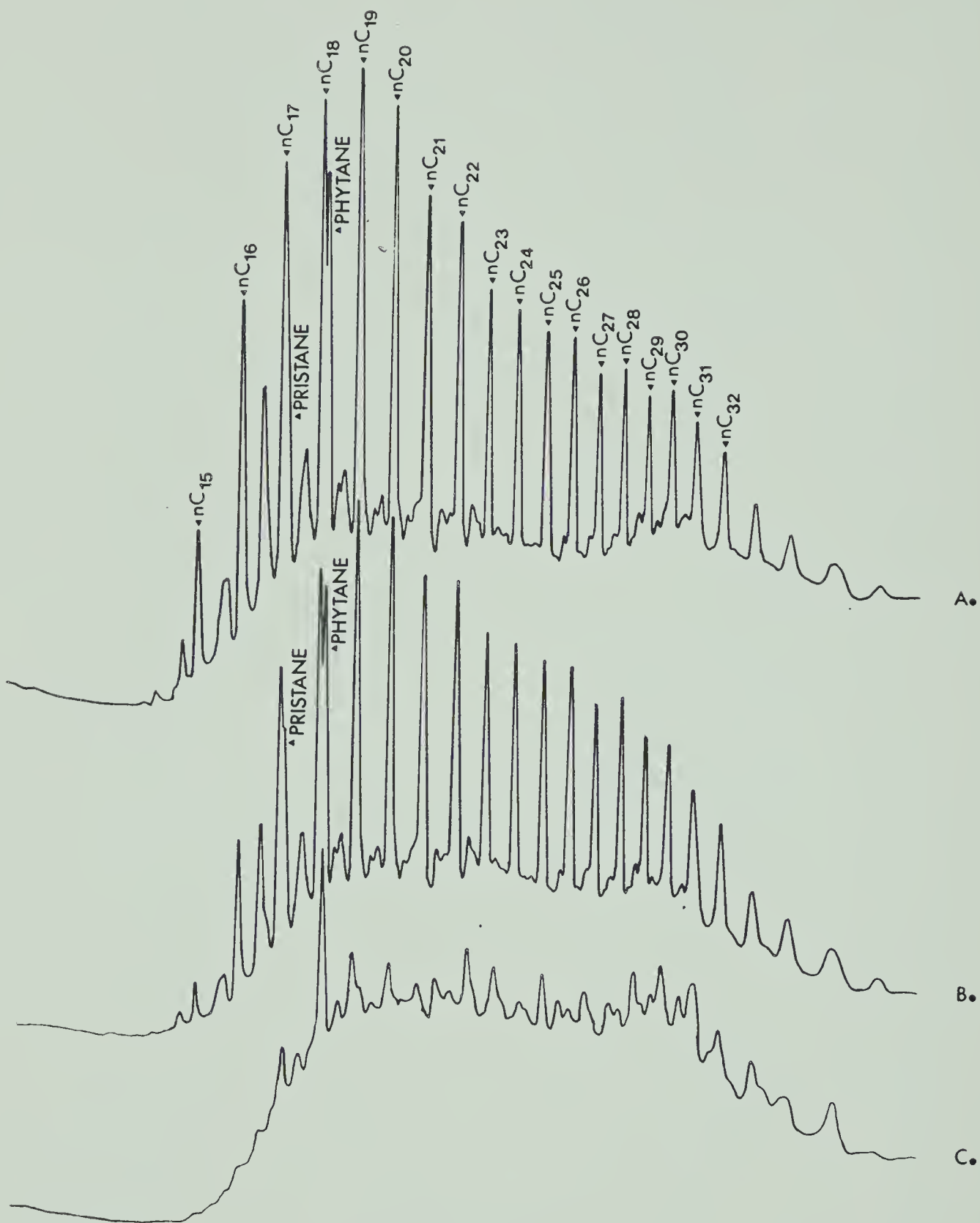


FIGURE 28

GLC ANALYSES OF RESIDUAL LOST HORSE HILL SATURATES
FOLLOWING METABOLISM BY BACTERIAL SOIL MIXTURES

- A. Lost Horse Hill control saturates.
- B. Residual Horse Hill saturates following degradation by the established mesophilic christmas tree soil mixture.
- C. Residual Horse Hill saturates following degradation by enrichment growth from neat soil inoculum.

SUMMARY AND CONCLUSIONS

A number of crude oils, taken from an extensive petroleum deposit in the province of Saskatchewan, have been subjected to metabolism by single soil isolates and mixtures of soil isolates at mesophilic and psychrophilic temperatures. All these oils can be reduced to a low quality residual product which closely resembles, physically and chromatographically, the poorest oil in the original deposit. These observations are coupled with the altered ^{32}S ; ^{34}S ratios, persistence of resistant isoprenoid features found both in naturally and artificially degraded oils, and evidence of extensive ground water wash in certain areas of the deposit. In total, they strongly implicate microorganisms as agents of secondary alteration of petroleums. It would have to be presumed that sufficient dissolved O_2 exists in ground water to allow the degradation to occur slowly, via an aerobic process, as no consistent evidence can be found to indicate significant anaerobic degradation.

The experiments carried out in this investigation also bring to bear certain points of interest important in terms of general bacterial metabolism rather than as evidence supporting an hypothesis of microbially-induced in situ petroleum degradation.

The first conclusion drawn from certain experiments in this investigation concerns the relative ease or difficulty

with which petroleum components can be utilized. It appears that component classification from the most easily degraded to the most refractive is roughly as follows: n-saturates, isoprenoids, isoparaffins, aromatics, benzene-soluble and insoluble asphaltenes, insoluble NSO's, soluble NSO's. It appears that the soluble NSO component is neither produced nor does it undergo significant catabolism in microbial systems. However, inducing growth from soil inoculations in solutions containing only NSO's as carbon substrate, could serve to change the order of the above classification.

A second conclusion centers around induction on a given petroleum, can successfully grow and metabolize other oils of a comparable or superior quality. If the bacterial mixture, previously induced on a high-quality oil, is faced with another petroleum with degraded or diluted n-saturate content, for instance, little or no growth will occur. Thus, it would seem feasible to establish a scale of petroleum rated from the highest to the lowest of saturate contents. In such a system, bacterial populations induced on a particular whole oil would probably emulsify and partially utilize most petroleum of superior quality, but would encounter problems with most of the relatively inferior oils.

A third point which seems apparent both from the results presented in the Introduction and the experiment described in Results and Discussion II, is the difficulty, if not the inability of bacteria to utilize oil anaerobically. It is

theoretically possible that microorganisms classified as aero-tolerant (including certain sulfate and sulfite reducers) may be able to utilize small amounts of O_2 in order to initiate hydrocarbon oxidation. Such a theory, however, requires experimental proof which has not yet been obtained.

An important point, in terms of pollution, centres around the experiment utilizing purified oil components as substrate for the mesophilic christmas tree soil mixture. Although only the residual saturate fraction stimulated growth, none of the other components appeared to be bactericidal toward the soil mixture, i.e. none of the initial viable counts decreased. Thus, though utilization of these components did not occur, it does not mean bacterial systems cannot tolerate or degrade the components if the proper procedures of enrichment or election are carried out.

Lastly, the positive utilization of n-saturates of greatly varying chain lengths is an indication of the extreme adaptibility of the two psychrophilic mixtures enriched for in this project. Overcoming the obvious problems of cold temperature solubility of paraffins indicates the good possibility of utilization of the other oil components, again if proper enrichment techniques are used to generate the organisms.

In closing, the obvious sacrificing of depth for the sake of scope in many of these experiments only yielded further problems which should be dealt with in greater detail in the future.

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APPENDIX I

SPECIAL PROBLEMS IN THE ESTIMATION
OF BACTERIAL GROWTH IN AQUEOUS - PETROLEUM SYSTEMS

Bacterial growth in any medium containing quantities of petroleum, or petroleum products, is a difficult task. This is aptly pointed out by Kuznetsova (1957), and Kuznetsov, Ivanov, and Lyalikova (1963).

Measurement of total growth by turbidometric (spectrophotometric) means is difficult to carry out when the medium also contains finely-dispersed oil droplets which remain stable within the medium for long lengths of time. Also, such measurements often tax the sensitivity of the spectrophotometer in cases where growth never exceeds $4 - 5 \times 10^7$ viable bacteria/ml. Such a small optical density is exceedingly prone to error.

Dry weights are often equally useless, especially when crude petroleum is used as the sole carbon and energy substrate. In this investigation, it was found that after a certain point in time, metabolism of such crudes rendered them more dense than the aqueous medium. Therefore, any attempts to centrifuge out the bacteria from a calibrated volume of liquid growth would often result in the bacteria and petroleum pelleting together, thus giving a falsely high weight.

Stable oil emulsions, as were formed in this project, interfered with any attempts of count the bacterial populations by means of a Coulter Counter. Therefore the only successful method of monitoring bacterial growth in this investigation was by plate count.

APPENDIX II

PROBLEMS IN RESIDUAL OIL RECOVERY FROM CULTURES

Since the purpose of this investigation was to study the changes imparted upon the petroleum by different growing cultures of bacteria, it was necessary to develop a repeatable method of oil recovery which was not prone to severe contamination by bacterial products.

It should be stated at this point that benzene is the only commonly used solvent in which whole petroleums are miscible. Benzene is not however a solvent of choice to put in the presence of bacterial cells. Cellular components, lipid components, and pigments all present problems of inconsistent extraction of cultures containing petroleum. Extraction of petroleum from cultures by centrifugation is an obvious solution of the problem. However, when petroleum has been used by bacteria as a growth substrate for a period of time, its specific gravity, as mentioned before, approaches and often exceeds that of water. Thus centrifugation only causes irreversible pelleting of the petroleum with the cells, a most undesirable situation when one is trying to obtain clean petroleum samples.

A third alternative, and one which was accepted as the method of choice, was to extract whole cultures with n-pentane. Although the asphaltenic component of petroleum is not soluble in n-pentane, it will align itself at the

n-pentane - water interface in a separatory funnel, the balance of the petroleum being in the n-pentane layer. Consequently, if one takes the n-pentane layer as well as the interfacial area, one can obtain the residual petroleum from a culture in a repeatable manner. Cellular disruption appears to be minimal with this method.

APPENDIX III

BACKGROUND CONCERNING GAS CHROMATOGRAPHIC ANALYSIS
OF SATURATE HYDROCARBONS

Numerous developments in gas chromatographic analysis in the past have been aimed at specific problems of resolution of a host of hydrocarbon components. The theoretical and practical reasons for these chromatographic techniques (McNair and Bonelli, 1969) have been refined and directed toward specific analysis of saturate fractions by Imperial Oil Producing Department personnel, (unpublished data).

Basically, resolution of 25 to 30 components within the saturate fraction of a topped oil was originally achieved using linear temperature-programming. Thus, by increasing the column temperature within the GLC unit at a constant rate, more component resolution could be achieved in a shorter column, than with a comparable column operated isothermally. Component retention times have been known to vary slightly more than in the case of isothermal operation. However, use of standard components (the preferred method in any case), as well as recognition of specific "landmarks" within the chromatographic profile eliminates this problem. Features such as isoprenoid peaks (e.g. pristane and phytane) within saturate GLC chromatographs, are easily recognized. For example, using an OV-1 column,

pristane (2,6,10,14-tetramethylpentadecane) and phytane (2,6,10,14-tetramethylhexadecane) elute immediately after heptadecane and octadecane respectively. Once these are noted, all the other normal paraffins are identified in linear ascending or descending order.

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